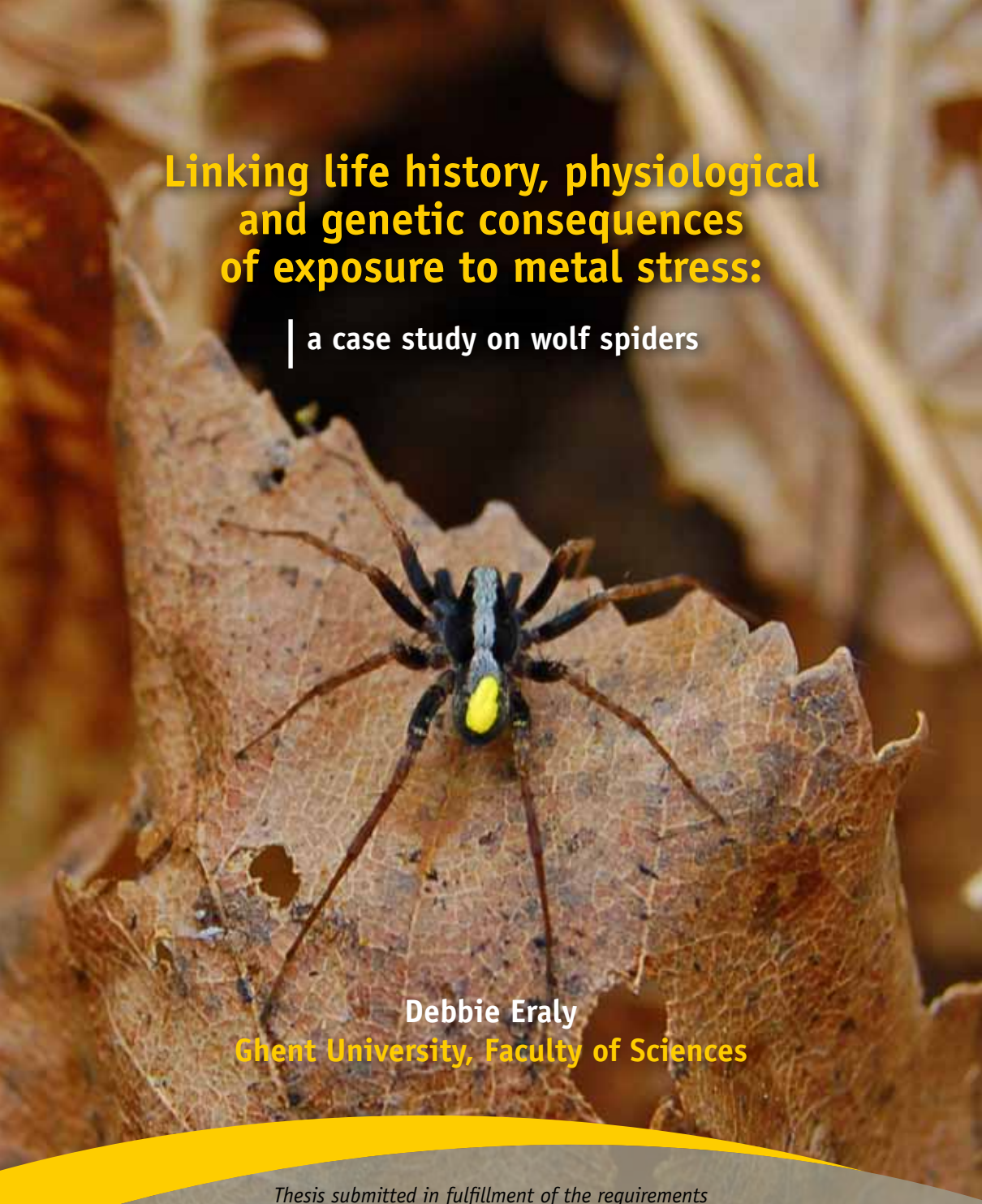
Linking life history, physiological and genetic consequences of exposure to metal stress:

a case study on wolf spiders



Debbie Eraly
Ghent University, Faculty of Sciences

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Sciences, Biology

Proefschrift voorgelegd tot het behalen van de graad van Doctor in de Wetenschappen, Biologie

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Everything is one, all is connected

wisdom of the Nuu-chah-nulth,
First Nation, West-Canada

GENERAL INTRODUCTION

The strength and direction of natural selection, the process underlying adaptive evolution, may differ in space and time, and may hence lead to adaptation and differentiation of populations both at the phenotypic and genotypic level (Posthuma & Van Straalen 1993; Kawecki & Ebert 2004; Reznick & Ghalambor 2001). Within this context local adaptation leads to a pattern where the resident genotype has a higher fitness in its own environment and is expected to perform worse outside its local habitat (Schluter 2001; Rasanen & Hendry 2008; Hereford 2009). Results of reciprocal transplant experiments indeed often show that genotypes are better adapted to their native environments than are genotypes from other populations, but there are examples of populations that have a relative fitness which is lower than that of foreign transplants (Hereford 2009). However, the ability to adapt to local conditions depends on different factors: the strength of the selective force, the level of standing genetic variation and the amount of gene flow and its relative strengths (Nosil 2008; Hedrick 2000). Processes such as genetic drift can limit the ability to adapt and can allow deleterious mutations to reach high frequencies within populations (Lynch *et al.* 1999). Although local adaptation is a common and well-studied phenomenon, more in depth studies are needed. Such studies are particularly needed in rapidly changing anthropogenic environments where populations may be exposed to different stressors at small spatial scales.

In this study, we address different topics in the context of natural populations exposed to metal stress. In a stressful environment defense mechanisms are expected to evolve and life history traits, like development time, survival, body size, clutch size, egg size and reproductive allocation are expected to differ between polluted and non-polluted populations. If these differentiations have a genetical basis, local adaptation is likely to evolve and could lead to reproductive isolation between divergent ecotypes.

1 METAL STRESS

1.1 Exposure to metals

The exposure of organisms to different pollutants, most often of human origin, is widespread and has been going on for decades. Yet, a large number of both plant and animal species still inhabit heavily polluted environments (Bengtsson *et al.* 1992; Posthuma & Van Straalen 1993). Metals are one of the most persistent pollutants with strong impacts on the fitness of plant and animal species, both directly and indirectly, and have been the subject of many empirical studies (Hunter *et al.* 1987a; Hunter *et al.* 1987b; Klerks & Levinton 1993; Posthuma & Van Straalen 1993; Harper *et al.* 1997; Bertin & Averbeck 2006; Burger 2008; Morgan *et al.* 2007; Lagisz & Laskowski 2008; Pook et al. 2009). They are generally considered to induce a strong selection pressure on natural populations as they are toxic at high concentrations, non-degradable and their detoxification requires additional energy expenditure (Calow 1991; Posthuma & Van Straalen 1993; Migula *et al.* 1997; Van Straalen & Hoffmann 2000; Reznick & Ghalambor 2001; Morgan *et al.* 2007). Therefore they provide an interesting natural experiment to study the mechanisms of micro-evolution.

As the bioavailability of metals in the environments is principally determined by the physical and chemical characteristics of the soil (Lock & Janssen 2001), soil concentrations often do not reflect exposure of metals to organisms (Hendrickx *et al.* 2004; Jung & Lee 2012). Instead, internal concentrations provide a more direct measure of the concentrations an organism is exposed to.

Metals can impair individual survival and growth in various ways. Direct physiological effects may occur when metals bind with essential molecules and alter their structure or function, which can lead to the inhibition of various enzymatic pathways and anti-oxidant activity. Indirect physiological effects may occur when reactive oxygen species are produced that disturb lipid structure, cause lipid peroxidation or apoptosis, depletion of cellular glutathione (GSH) or inhibition of various enzymatic pathways and anti-oxidant activity. This is caused by the binding of metals to

essential molecules, which affects their structure or function (Viarengo & Nott 1993; Lukkari *et al.* 2004; Wilczek *et al.* 2004; Wilczek 2005; Bertin & Averbeck 2006). By eliciting energetically expensive tolerance mechanisms, they can also affect the energy budget of the organism (Calow 1991; Van Straalen & Hoffmann 2000). An often neglected, yet equally important, effect of metal pollution is the biological impact through interconnected ecological effects. For instance, changes in vegetation structure, in community composition or reduction in prey availability may result in a reduction in fitness due to changes in intra- and interspecific competition for critical resources and/or changes in predation risk. Such effects often alter the energy budget of organisms, and hence, their investment in reproductive output or even survival (Vanhook & Yates 1975; Hunter *et al.* 1987b; Posthuma & Van Straalen 1993; Lock *et al.* 2003; Reznick & Ghalambor 2001). Population-level changes may, in turn, cause effects at community level, especially if highly sensitive species are keystone members, and as such may affect critical ecosystem functions (Coughtrey *et al.* 1979; Hunter *et al.* 1987a; Tyler *et al.* 1989; Read *et al.* 1998; Nahmani & Lavelle 2002; Creamer *et al.* 2008; Clements & Rohr 2009).

1.2 Defense mechanisms

Yet, organisms have several mechanisms by which they can protect themselves against the adverse effects of heavy metals, such as intake avoidance, reduced uptake, decreased accumulation, increased excretion, detoxification or sequestration in a non-toxic form (Bengtsson *et al.* 1992; Posthuma & Van Straalen 1993; Viarengo & Nott 1993; Wilczek *et al.* 2004; Xie & Klerks 2004; Bahrndorff *et al.* 2006; Morgan *et al.* 2007). Some enzymes and molecules can increase tolerance, with a major role for glutathione, heat shock proteins and metallothionein-like proteins. After they bound to the metals, they are often stored in intracellular mineral granules (Metal-Rich Granules, MRG), which are insoluble and thus physiologically unavailable (Mason & Jenkins 1996; Kohler *et al.* 1999; Knigge & Kohler 2000; Park *et al.* 2001; Lukkari *et al.* 2004; Wilczek *et al.* 2004; Wilczek 2005; Santiago-Rivas *et al.* 2007; Wilczek *et al.* 2008).

In this plethora of defense mechanisms we focus on MetalloThionein-Like Proteins. MTLPs are known to play an important physiological role in defense against metal stress. MTLPs are non-enzymatic proteins with a low molecular mass and high affinity for metals. Their high cysteine content consisting of sulphydryl-groups directly bind metals in insoluble fractions (Brown 1982; Mason & Jenkins 1996; Viarengo & Nott 1993; Dallinger 1996;(Roesijadi 1996; Nordberg 1998; Park *et al.* 2001; Kohler 2002; Lukkari *et al.* 2004; Amiard *et al.* 2006; Santiago-Rivas *et al.* 2007; Wilczek *et al.* 2008; Janssens *et al.* 2009). While MTLPs sometimes are constitutively expressed i.e. independent of a particular environmental factor, and thus even in the absence of metal exposure (basal MTLP; Petering & Fowler 1986), their synthesis can also be induced upon exposure to metals (Dallinger 1996; Pedersen *et al.* 1997; Viarengo *et al.* 1999; Hensbergen *et al.* 2000; Lukkari *et al.* 2004; Van Campenhout *et al.* 2008). The latter has been shown in the springtail *Orchesella cincta* and related to cis-regulation of the gene (Sterenborg & Roelofs 2003; Roelofs *et al.* 2006) together with trans-acting factors (Janssens *et al.* 2009). They have therefore repeatedly been proposed to constitute a highly suitable biomarker for metal exposure (Hopkin 1989; Viarengo & Nott 1993; Roesijadi 1996; Viarengo *et al.* 1999). Induction levels have been shown to vary among individuals, species, metals, and biotic and abiotic condition (Amiard *et al.* 2006*).* MTLPs were originally discovered in mammals and have thereafter been observed and studied in a wide range of marine and terrestrial invertebrates including collembolans, nematods, isopods, gastropods, earth worms, *Drosophila* (Viarengo & Nott 1993; Dallinger 1996; Dallinger *et al.* 2000; Znidarsic *et al.* 2005; Carpene *et al.* 2007; Hughes & Sturzenbaum 2007; Janssens *et al.* 2009). It can be expected that different detoxification mechanisms interact, e.g. with metals initially bound to MTLPs subsequently being redistributed into Metal Rich Granules (Mason & Jenkins 1996; Wallace *et al.* 2003).

1.3 Life history theory and stress

Life history theory aims at explaining how traits that are closely related to individual fitness like size and age at maturity, fecundity and propagule size are connected and which environmental factors determine the strength

and direction of selection (Sibly & Calow 1989; Stearns 1992). Life histories are shaped by the interaction of extrinsic and intrinsic factors. The extrinsic factors are ecological impacts on survival and reproduction, the intrinsic factors are tradeoffs among life history traits and lineage-specific constraints on the expression of genetic variation (Stearns 2000). Classical life history theory, summarized in Roff (1992) and Stearns (1992), is based on optimization models. The concept of trade-offs forms an integral part of life history theory. The assumption is that an individual has a limited amount of energy available to invest into three basic functions: growth, somatic maintenance, or reproduction (Fox & Czesak 2000; Van Oers *et al.* 2005).

An important application of life history theory is evolution in stressful environments (Bengtsson *et al.* 1992; Posthuma & Van Straalen 1993; Posthuma *et al.* 1993; Shirley & Sibly 1999; Hendrickx & Maelfait 2003; Lagisz & Laskowski 2008). Generally, stress is defined as a condition that reduces Darwinian fitness by reducing survival, fecundity, time between life cycle events, etc (Sibly & Calow 1989; Rozen 2006). For several species, differences in life-history traits, like development time, survival, body size, clutch size, egg size and reproductive allocation between polluted and non-polluted populations have indeed been demonstrated (Posthuma & Van Straalen 1993; Posthuma & Van Straalen 1993; Shirley & Sibly 1999; Hendrickx *et al.* 2003).

Age of maturation is one of the factors to be expected to change when facing stress. Early maturation has the advantage of a shorter generation time and a higher probability of surviving to adulthood. Maturing at a later age allows an organism to acquire more resources until adulthood, leading to a larger size at maturity and greater fecundity, since fecundity often increases with body size and could lead to the production of higher-quality offspring (Stearns 2000). In organisms living in a stressed environment, early maturation may be favored to reduce the probability of death between birth and maturity. On the other hand, as energy might be more limited under stressful conditions, maturation might have to be postponed (Sibly & Calow 1989; Colvin & Gatehouse 1993; Winterer & Weis 2004). Other important and extensively studied traits are the number and size of

eggs and offspring. As resources invested in reproduction can be divided into either many small progeny or less but larger progeny, there may occur a trade-off between the number and size of progeny (Smith & Fretwell 1974; Fox & Czesak 2000). Under different environmental conditions, the relation between progeny size and progeny fitness will vary, leading to different optimal progeny sizes (Fox & Czesak 2000). Smaller eggs tend to result in smaller hatchlings that grow into smaller instars and have lower juvenile survival. This implies that under conditions where initial size has little effect on offspring fitness, females are expected to produce a large number of small eggs (Rasanen *et al.* 2005). Progeny hatching from larger eggs in general survive better, mature earlier and develop faster and are thus expected to be better able to face environmental stresses such as environmental pollutants. Hence, under these circumstances a small number of large eggs is expected (Smith & Fretwell 1974; Hendrickx *et al.* 2003; Fox & Czesak 2000; Sibly & Calow 1986; Stearns 1992; Tamate & Maekawa 2000).

When organisms are exposed to stress, different physiological defense mechanisms are deployed.These are generally expected to reduce the energy available for growth and reproduction (Southwood 1988; Sibly & Calow 1989; Calow 1991; Donker *et al.* 1993a; Posthuma & Van Straalen 1993; Zera & Harshman 2001). However, in some species of isopods and Collembola, individuals inhabiting polluted habitats are more vigorous. This could be explained bydirect selection on life-history characteristics related to fitness (Posthuma & Van Straalen 1993). Moreover, and contrary to what is generally assumed, various tolerance mechanisms are actually modifications of energetically cheap metal-regulation mechanisms (Calow 1991; Posthuma & Van Straalen 1993; Van Straalen & Hoffmann 2000). All together this puts into questions to what degree metal defense impacts the energy available for other processes in an exposed individual.

2 METAL ADAPTATION

2.1 Adaptation

Local adaptation is the consequence of environmental heterogeneity causing different selective forces in different places that leads to the evolution of traits that provide an advantage of the local individual compared to immigrants (Kawecki & Ebert 2004; Lenormand 2012). This results in different ecotypes, a dynamic concept that refers to groups of populations, distinguished by a composite of variation in different traits and allele frequencies across loci over space (Lowry 2012). Taking into account the environmental dependence of trade-offs, local adaptation to one environment may also cause lower relative fitness in alternative environments, though this is not necessarily so. The experimental evidence for fitness trade-offs is mixed, and shows that adaptation and specialization can also evolve in the absence of trade-offs (Fry 1996; Hereford 2009). Reciprocal transplant experiments allow to directly test of the hypothesis of costs of adaptation or fitness trade-offs (Schluter 2001; Hereford 2009). A higher fitness in the native environment indicates that adaptation to one environment comes at a cost of adaptation to other environments. Superior fitness of one population in both environments indicates adaptation without a fitness trade-off. The survey of the literature on all taxa by Hereford (2009) suggests that local adaptation is common and that, on average, a local population has 45% greater fitness than a foreign population and almost half of the comparisons showed evidence of a trade-off. Costs of adaptation do not appear to be strong enough to prevent simultaneous adaptation to multiple environments. Weak costs of adaptation may explain why the predictions of theory relating evolution of specialization to costs of adaptation are frequently not met (Hereford 2009).

The important role for environment-dependent micro-evolution is shown by growing empirical evidence (Schluter 2001; Levin 2000; Lexer & Fay 2005; Parsons 1995; Hoffmann & Hercus 2000; Reznick & Ghalambor 2001; Rasanen & Hendry 2008). Providing evidence for local adaptation is still challenging because also reflects ecological constraints (Kawecki & Ebert 2004). First, because of the costs involved in tolerance linked to energetic

constraints, and other trade-offs as described in the paragraph above (Shirley & Sibly 1999; Van Straalen & Hoffmann 2000; Kawecki & Ebert 2004; Morgan *et al.* 2007). Second, because of the homogenizing effect of gene flow (Reznick & Ghalambor 2001). The co-existence of adapted and non-adapted populations necessitates a strong restriction on gene-flow (Rasanen & Hendry 2008) or ecological by-product mechanisms should evolve (see §3). For strong stressors, adaptation can occur even in the presence of high gene flow levels, leading to isolation by adaptation as opposed to isolation by distance (Nosil *et al.* 2009; Nosil *et al.* 2007; Dhuyvetter *et al.* 2007; Kawecki & Ebert 2004).

Because metals are persistent and strong stressors, they are one of the best studied stressors (Hopkin 1989; Donker & Bogert 1991; Bengtsson *et al.* 1992; Aziz *et al.* 1999; Jordaens *et al.* 2006; Pauwels *et al.* 2006) and adaption has been shown in different groups of organisms (Klerks & Weis 1987; Postma *et al.* 1995; Dallinger 1996; Martinez & Levinton 1996; Mouneyrac *et al.* 2002; Sterenborg & Roelofs 2003; Morgan *et al.* 2007; Hendrickx *et al.* 2008; Roelofs *et al.* 2009 ; Costa *et al.* 2012) and is also found to occur quickly (Antonovics *et al.* 1971; Posthuma & Van Straalen 1993; Carroll *et al.* 2007). To proof populations to be locally adapted to a stressor, persistence in itself is not enough, they should also show differential survival compared to non-adapted conspecifics and show genetic differences (Kawecki & Ebert 2004).

In a further stage, local adaptation could lead to ecological speciation, when divergent selection on traits between populations or subpopulations in contrasting environments leads to the evolution of reproductive isolation (Schluter 2001; Rundle & Nosil 2005; Roff & Fairbairn 2007; Hereford 2009; Lenormand 2012; dicussed in more detail in § 3). In the context of ecological speciation, reproductive isolation can evolve as a result of ecologically-based divergent selection between populations through different mechanisms (Lande & Kirkpatrick 1988; Nosil *et al.* 2003; Funk *et al.* 2006; Schluter 2001; Mckinnon *et al.* 2004; Schwartz & Hendry 2006, see §3 in this chapter).

2.2 Phenotypic plasticity and acclimation

Moreover, populations can also have the ability to physiologically acclimate. Acclimation is defined as increased tolerance acquired at the individual level through long term exposure to sublethal concentrations and can be considered a form of phenotypic plasticity (Bengtsson *et al.* 1992; Posthuma & Van Straalen 1993; Belfiore & Anderson 1998). Phenotypic plasticity is the tendency of a particular genotype to produce different phenotypes under different environmental conditions. It allows individuals to deal with environmental unpredictability and can be favoured over genetic adaptation, though it also has its costs and limits and can be maladaptive (Thibert-Plante & Hendry 2011). Though theoretically the concepts of adaptation and acclimation are clearly distinct, it is not always easy to differentiate between increased resistance due to physiological acclimation and genetic adaptation, and both mechanisms have been found to be present in metal exposed populations (Antonovics *et al.* 1971; Klerks & Levinton 1989; Bengtsson *et al.* 1992; Posthuma & Van Straalen 1993; Shirley & Sibly 1999; Knapen *et al.* 2004; Xie & Klerks 2004). Moreover, phenotypic plasticity in tolerance can also be a genetic adaptation, with the tolerance mechanism only put into action when exposed to heavy metals (Posthuma & Van Straalen 1993; Roelofs *et al.* 2009). When applied to MTLP, if historically metal-polluted populations are genetically adapted, we expect higher constitutive MTLP concentrations in individuals originating from these populations compared to individuals from reference populations (as in Timmermans *et al.* 2005). If individuals show acclimation, instead, MTLP levels are expected to be inducible upon exposure (as in: Dallinger 1996; Roesijadi 1996; Pedersen *et al.* 1997; Nordberg 1998; Viarengo *et al.* 1999; Hensbergen *et al.* 2000; Lukkari *et al.* 2004; Van Campenhout *et al.* 2008). Finally, in case of adaptive plastic acclimation, individuals from historically polluted populations are expected to show a larger increase in MTLP production upon exposure than individuals from unpolluted reference populations.

To discriminate among these underlying mechanisms, studies on offspring, preferably originating from reciprocal crosses, that are bred under common garden conditions are necessary (Kawecki & Ebert 2004; Lagisz & Laskowski 2008; Hereford 2009).

3 ADAPTATION AND SEXUAL SELECTION

One of the possible consequences of local adaptation with potentially great evolutionary impact is the evolution of reproductive isolation between adapted and non-adapted populations, both through direct and indirect processes (Ortigosa & Rowe 2002; Hunt *et al.* 2005; Fisher & Rosenthal 2006; Lenormand 2012). Local populations that are exposed to different environmental conditions may also face different sexual selection regimes, which may ultimately result in reproductive barriers among populations. This results in ecological speciation, a theory which states that reproductive isolation evolves as a result of ecologically-based divergent selection between populations (Schluter 2001). Currently, the theory of ecological speciation leading to reproductive isolation is well established due to a growing number of theoretical and empirical studies, though it remains a complex and challenging subject(e.g. Schluter 1996; Carroll *et al.* 1997; Losos *et al.* 1997; Foster *et al.* 1998; Orr & Smith 1998; Dieckmann & Doebeli 1999; Barton 2001; Rolan-Alvarez *et al.* 2004; Nosil *et al.* 2005; Vines & Schluter 2006; Funk 1998; Vines & Schluter 2006; Jennions & Petrie 1997; Funk *et al.* 2006 ; Rice *et al.* 2011; Lenormand 2012). Though local adaptation is a prerequisite for ecological speciation, it is not sufficient (Berner & Hendry 2009; Hendry 2009 ; Nosil, *et al.* 2009; Labonne & Hendry 2010; Thibert-Plante & Hendry 2011). While abiotic environmental differences are the most straightforward sources of divergent selection, some forms of sexual selection and ecological interactions may trigger divergent selection as well (Rundle & Nosil 2005). The appeal of this concept is that the main focus is on the action of natural selection and its driving mechanisms, instead of on the geographical arrangement of populations as is traditionally the case (Via 2001).

Two main mechanisms may lead to assortative mating under ecological divergence:

- First, mate choice may diverge if adaptation causes phenotypic traits on which mate choice is based to diverge due to natural selection. The is called the "by-product mechanism" (Funk 1998; Vines & Schluter 2006). It can be due to a functional relationship or through the genetical mechanisms of pleiotropy and linkage disequilibrium (Verrell 1999; Schluter 2001; Turelli et al. 2001). A growing number of theoretical as well as empirical studies demonstrate that divergent natural selection can affect mating behavior and as such cause reproductive isolation between the diverged populations in the face of gene flow (reviewed in Hey, 2006 and Nosil, 2008). A clear example of ecological speciation by the by-product mechanism is size-assortative mating (Mckinnon et al. 2004). Another is a shift in reproductive timing, possibly linked to energetic constraints (Winterer & Weis 2004). This process has been widely studied in plants but less so in animals, despite their presumed effects on population dynamics and reproductive isolation between stressed and reference populations (Fox 2003; Winterer & Weis 2004; Weis et al. 2005).
  This also relates to condition-dependence of male mating signals, implying males not adapted to the stressor having a lower fitness in the stressed habitat (Greenfield & Rodriguez 2004; Kotiaho 2000; Parri et al. 2002). But the ecological condition and state of the choosing individual also affects mate preference or its strength. Energetic limitations which could make mating more costly under stress, increasing the pressure for being more selective when choosing a mate. Theoretical models predict individuals to be less choosy when the cost of choice increases (Real 1990; Pomiankowski et al. 1991). Thus condition-dependent effects have the potential to control the direction and strength of sexual selection (Archard et al. 2006).
  Environmental differences like predation risk, exposure to pollutants, sex ratio, density and food availability can also affect mating behavior through phenotypic plasticity (Rowe et al. 1994; Jennions & Petrie 1997; Thibert-Plante & Hendry 2011). Through plastic responses to environmental differences phenotypic novelties can arise. The

continuous expression of these environmentally induced traits can further be integrated into the genetic architecture of an orgtanism, a process called genetic accommodation (the change in gene frequencies because of the act of selection in the subpopulationWest-Eberhard 2005).Hence, based on this theory, it can be expected that environmentally induced changes in sexually selected traits may ultimately lead to the evolution of reproductive barriers.

- Second, when populations are in secondary contact, natural selection may also lead to prezygotic isolation if selection occurs to reduce the formation of unfit hybrids; a process called reinforcement (Smadja & Butlin 2006). The reduced hybrid viability can be due to ecological selection, genetic incompatibility or sexual incompatibility (Doebeli & Dieckmann 2000; Schluter 2001).

The direct action of sexual selection is a powerful force in generating assortative mating. Ecologically-based sexual selection involves spatial variation in selection on secondary sexual traits, on mating signal transmission or divergent selection on sensory systems (Turelli *et al.* 2001). Though, it can also occur through non-ecological forces as drift, the occurrence of different mutations and through interactions between the sexes as Fisher's runaway selection and sexual conflict (Schluter 2001).

Environmental effects on mate choice have been studied within the context of signal reliability and courtship behavior (Andersson 1994; Parsons 1995; Jennions & Petrie 1997; Kotiaho 2000; Kotiaho *et al.* 1998; Uetz *et al.* 2002). In general, females are expected to choose males on the basis of traits that reflect their quality and condition. Conditional handicap models thereby predict that sexual traits are reliable indicators of mate quality since they are condition-dependent and costly to produce (Rowe & Houle 1996; Andersson 1994; Johnstone 1995; Parri *et al.* 1997; Kotiaho 2000; Uetz *et al.* 2002; Ahtiainen *et al.* 2006; Johnstone 1995). When environmental factors vary and affects male condition, this mechanism could lead to environmental dependent mate choice. In the wolf spiders *Hygrolycosa rubofasciata* and *Schizocosa ocreata,* where males show elaborate courtship display behavior and secondary sexual traits, evidence

for this hypothesis is present (Parri *et al.* 1997; Kotiaho 2000; Uetz *et al.* 2002; Ahtiainen *et al.* 2006). This, however, takes an implicit male angle, and it is less well understood how ecological variation may affect both the strength and direction of female mate choice behavior (Jennions & Petrie 1997; Archard *et al.* 2006; Ortigosa & Rowe 2002). Condition-dependence in female mate choice is an important factor since this costly behavior is also expected to depend on her reproductive status (Parker 1983; Hunt *et al.* 2005; Jennions & Petrie 1997; Janetos 1980). The cost of mate choice depends on the time and energy spend on evaluating and mating that cannot be spend on foraging and increases the vulnerability to predators.

In female mate choice two aspects can be distinguished i.e. female responsiveness and preference. Responsiveness is defined as the willingness to engage into mating while preference is a measure of the male traits that are favored and the choosiness or willingness to invest in mate assessment (Jennions & Petrie 1997). Lower responsiveness can weaken sexual selection if this increases the likelihood of random mating, while it can strengthen sexual selection if it increases the threshold for male attractiveness. Some theoretical models predict individuals to be less choosy when they are of low quality, because they have a lower probability or survival or of meeting a mate in good condition, or when the cost of choice increases (Real 1990; Pomiankowski *et al.* 1991; Hunt *et al.* 2005; Hingle *et al.* 2001; Clark *et al.* 1997). On the other hand, lower energy availability also makes it more costly to mate and could increases the need to make the right choice, leading to a stronger selectivity (Rowe *et al.* 1994; Fisher & Rosenthal 2006; Ortigosa & Rowe 2002) or could increase the differences between males, making choice more reliable (Clark *et al.* 1997). Environmental conditions affecting resource availability could thus alter the strength of sexual selection by altering the optimal mating rate, but could also lead to different phenotypes to be sexually selected thereby possibly reinforcing adaptive divergence (Vines & Schluter 2006). Condition-dependence is not only relevant for within population differences but also for differences between populations since different environments are very likely to result in differences in individual condition. Recent empirical studies have shown that female condition dependence can affect mate choice through both responsiveness and preference (Wilder &

Rypstra 2008; Hebets *et al.* 2008; Tigreros & Switzer 2008; Burley & Foster 2006; Fisher & Rosenthal 2006; Hunt *et al.* 2005).

Body size is an important trait of an individual that is correlated with physiological characteristics and fitness, and often regarded as an important factor in mate choice. Generally larger females are chosen because of fecundity selection or increased quality of her offspring, while larger males often do better in male-male competition (Andersson 1994; Blanckenhorn 2000). Since size is highly environmentally dependent, the optimal size is likely to vary when the environment does and as a consequence so is mate choice. Moreover, animals are often shown to mate assortatively according to size (Crespi 1989; water striders: Rowe *et al.* 1994, Arnqvist *et al.* 1996 and Ortigosa & Rowe 2003; beetles: Harari *et al.* 1999; sticklebacks: Schluter & Nagel 1995; jumping spider: Hoefler 2007 and the funnel-web spider: Masumoto 1999). The mechanisms leading to it are diverse, going from mate availability, the importance of size in overcoming female resistance, male-male competition, physical constraints in the mating apparatus or because of the loading capacity (when the female is carrying the male when copulating) and active mate choice (Crespi 1989; Harari *et al.* 1999). For mate choice to lead to size-assortative mating, males and females should prefer large mates and in competition for large mates, larger individuals should be more likely to win (Rowe & Arnqvist 2002; Harari *et al.* 1999). If differences in environments or population characteristics between populations result in differences in size or the strength of assortative mating, this mechanism could enforce reproductive isolation (Crespi 1989).

In a wide range of animal groups several empirical studies already reported changes in different aspects of both male and female mating behavior due to environmental stressors or resource availability cfr. female receptivity and preference in water striders (Ortigosa & Rowe 2002, crickets Hunt et al. 2005), guppies (Syriatowicz & Brooks 2004 and Archard *et al.* 2006), swordtail fish (Fisher & Rosenthal 2006), sticklebacks (Bakker *et al.* 1999), stalk-eyed flies (Hingle *et al.* 2001 and Cotton *et al.* 2006), cockroaches (Clark *et al.* 1997) and *Drosophila*, (Fasolo & Krebs 2004); signal reliability in bank voles (Hunt *et al.* 2005) and wolf spiders (Kotiaho 2000); male

courtship behavior in *Drosophila* (Fasolo & Krebs 2004) and cockroaches (Clark *et al.* 1997).

Environmental differences between populations could thus lead to reproductive isolation in absence of geographical barriers. The interaction between adaptive divergence and gene-flow in the diversification of populations is currently much debated in evolutionary studies (Rasanen & Hendry 2008; Nosil 2008; Schluter 2001; Niemiller *et al.* 2008; Coyne & Orr 2004; Hey 2006). A growing number of theoretical as well as empirical studies demonstrate that divergent natural selection can affect mating behavior and as such cause reproductive isolation between the diverged populations in the face of gene flow reviewed in Hey (2006) and Nosil (2008).

The mechanisms driving ecological speciation are still far from being fully understood and the evidence for ecological speciation is still incomplete. Further clarification of the genetic mechanisms underlying and linking ecological divergence and reproductive isolation is urgently needed. Tests in nature and the laboratory, between populations, complemented with other than model systems are necessary (Orr & Smith 1998; Schluter 2001; Rundle & Nosil 2005; Foster *et al.* 1998).

4 ECOTOXICOGENETICS

For populations to be able to adapt to changing conditions, sufficient genetic variation for natural selection to act upon should be present on the one hand (Frankham 2005; Barrett & Schluter 2008; Schluter & Conte 2009) and gene flow between non-exposed populations should be restricted (Slatkin 1993) though not necessarily absent (Hey 2006; Nosil 2008). The effects of metal stress on genetic differentiation and diversity on a genome wide scale have recently been studied extensively (Bickham *et al.* 2000; Van Straalen and Timmermans 2002; Morgan *et al.* 2007; Roelofs, Janssens, *et al.* 2009; Costa *et al.* 2012). Theoretical and molecular advances in population genetics allow to search and even identify loci subjected to divergent selection, metal polluted populations provide interesting cases

for studying natural selection in action (Van Straalen & Timmermans 2002; Joost *et al.* 2007; Foll & Gaggiotti 2008; Williams & Oleksiak 2008; Hohenlohe *et al.* 2010; Roelofs *et al.* 2010; Van Straalen & Feder 2012).

Different, mutually non-exclusive, patterns and processes can be expected in a structure of populations of the same species facing different levels of stress.

First, some loci on which selection acts and those in close linkage (outlier loci) could diverge, while the remainder of the loci will be influenced by classical demographics (e.g. bottlenecks, founder effects, inbreeding leading to a pattern of outlier loci or genomic islands of divergence ; Nosil *et al.* 2007; Joost *et al.* 2007). All the loci across the genome are expected to respond similarly to demography and neutral history of populations, whereas only a few loci, or a set of closely linked loci, are affected by natural selection (Lewontin 2002; Manel *et al.* 2003). These outlier loci are potential signatures for adaptation and can provide more insight in adaptation mechanisms and allow to identify key genes in microevolutionary process of speciation (Bonin *et al.* 2006; Joost *et al.* 2008).

Second, long periods of ongoing divergent selection can result in fitness reduction of hybrids due to recombination and maladaptation. This could result in selection for reproductive isolation i.e. reinforcement or selection against hybrids. In this case, gene flow among ecologically divergent populations becomes restricted, while exchange of genes remains possible between populations subjected to the same selection pressure. Under this scenario, we expect that differentiation between ecotypes will not be restricted to those loci that are directly involved in adaptation, but even for neutral genes scattered throughout the genome (cfr. Wu 2001, the genetic view of speciation). Overall genetic variation, in the metapopulation will thus not be expected to decrease (Van Straalen & Timmermans 2002).

Third, if selection against maladapted individuals is very strong, only a few individuals will be able to survive in stressful environments leading to population bottlenecks and founder events (Van Straalen & Timmermans

2002). This can lead to a pattern of genome wide genetic erosion that is reflected in an overall decrease in genetic diversity (Dibattista 2008). Since genetic variation ensures the potential for future adaptations, a decrease could limit this potential and even long-term survival (Schluter & Conte 2009; Barrett & Schluter 2008). Since metalliferous sites often are of small size and geographically isolated within an unpolluted landscape matrix this mechanism is likely to occur (Wolf 2001; Van Straalen & Timmermans 2002; Morgan *et al.* 2007; Fratini *et al.* 2008; Lopes *et al.* 2009).

5 STUDY SPECIES

5.1 The wolf spider family

The topics discussed above are studied on two representatives of the spider family, Lycosidae (wolf spiders). Spiders have a key role in ecosystems and, as predators and polyphages, are macroconcentrators of metals (Maelfait & Hendrickx 1998; Heikens *et al.* 2001; Tojal *et al.* 2002; Jung *et al.* 2008). Contrary to insects, which mainly excrete metals (Van Straalen *et al.* 1987; Janssen *et al.* 1991; Lindqvist & Block 1995; Kramarz 1999), the detoxification mechanism in spiders is principally based on storage (Janssen *et al.* 1991; Kramarz 1999). Spiders are strong accumulators of metals and their internal concentration reflects the exposure throughout their lifetime (Hendrickx *et al.* 2003).

Ecotoxicological properties of spiders appear to differ profoundly between spider families. For instance, Lycosid spiders generally have higher Cd, Zn, Pb and Cu burdens than web-building spiders captured at the same site, likely as a result of their more direct contact with polluted soil, their active hunting strategy and their type of prey, mainly Diplopoda, Diptera larvae, Collembola and Isopoda, species groups that are known to store heavy metals (Vanhook & Yates 1975; Nyffeler & Benz 1981; Larsen *et al.* 1994; Wilczek & Migula 1996; Hendrickx *et al.* 2004; Wilczek *et al.* 2008; Jung *et al.* 2008). Studies on other Lycosids, *Pardosa amentata* (Wilczek & Babczynska 2000) and *Pirata piraticus (*Hendrickx et al. 2002)*,* showed that their internal cadmium concentration increased linearly during exposure

and bioelimination rates are generally low or even estimated to be zero for Cd, Zn and Pb. Lycosid spiders appear to be capable to live in heavily metal-polluted habitats (Larsen *et al.* 1994; Wilczek & Migula 1996; Baker *et al.* 2001; Wilczek *et al.* 2003; Hendrickx *et al.* 2004; Wilczek *et al.* 2004; Wilczek 2005; Jung *et al.* 2007; Jung *et al.* 2005; Chen *et al.* 2011; Jung & Lee 2012).

Detoxification mechanisms in spiders are mainly based on storage in intracellular granules (Breymeyer & Odum 1969; Vanhook & Yates 1975; Brown 1982; Hopkin & Martin 1985; Hopkin 1989; Janssen *et al.* 1991; Wilczek & Babczynska 2000) or temporary mineral concretions "spherites", (Ludwig & Alberti 1988). Higher concentrations of metals are measured and probably stored in the midgut, or hepatopancreas, that has a low metabolic rate and may serve as a protective barrier for other sensitive organs such as the gonads (Ludwig & Alberti 1988; Hopkin 1989; Posthuma & Van Straalen 1993; Foelix 1996; Wilczek & Babczynska 2000).

As far as we are aware, metallothioneins in spiders have been studied in two other wolf spider species, the closely related *P. lugubris* (Wilczek 2005) and *Xerolycosa nemoralis* (Wilczek *et al.* 2008) through flow cytometry based on an immunological reaction and on three species of other spider families (Babczynska *et al.* 2011). *P. lugubris* from a polluted site exposed to metals in the laboratory had on average twice the number of MTLP-positive cells compared to those from a reference population. For the midgut gland no significant differences in the number of MTLP-positive cells was present, probably because detoxification of metals mainly depends on storage in intracellular granules, which is less energy-consuming than the production of MTLP (Wilczek 2005).

Besides the characteristics mentioned above, Lycosids are particularly well suited for our research for the following reasons: (i) they often reach high densities in severely polluted ecosystems and thus are able to cope with metal stress (Wilczek & Babczynska 2000; Wilczek *et al.* 2005; Jung *et al.* 2008); (ii) because of their conspicuous behavior they are easy to locate and observe in the field; (iii) they occur at high densities; (iv) females carry their eggs in a cocoon attached to their spinnerets so female reproductive output clutch mass, number of eggs and egg volume can

easily be determined and related to female characteristics; (v) they usually are structured in clearly delimited populations because of their specific habitat demands; (vi) they are relatively easy to breed in the laboratory which allows for laboratory experiments.

5.2 Pirata piraticus

The wolf spider *Pirata piraticus* is a generalist species of wet, open habitats that can be particularly common in tidal marshes with extensive reed fringes but also inhabits heavily polluted sites close to industrial plants (Hendrickx et al. 2003a). It has an annual life cycle with males becoming adult at the end of April and the beginning of May, just before the females do (Toft 1979). The species can easily be bred under laboratory conditions which makes it suitable for eco-evolutionary study. Under natural conditions, adult females produce one or two egg sacs May-August with larger females breeding earlier in the season and showing larger clutch volumes and masses (Hendrickx *et al.* 2003b). Contrary to most other wolf spiders that are used for studies on courtship behavior (e.g. Kotiaho *et al.* 1996; Hebets & Uetz 2000; Töpfer-Hofmann *et al.* 2000), *P. piraticus* male courtship behavior is short, less conspicuous and does not include pronounced leg or abdomen movements. Males also lack obvious secondary sexual traits.

Picture 1 | Female Pirata piraticus with egg cocoon kept in the laboratory in a container with plaster.

Previous research on *Pirata piraticus* showed that populations inhabiting metal polluted sites exhibited life history characteristics that confirm the reduction in resource acquisition theory and key life history traits to be negatively related with average metal body burden (Hendrickx *et al.* 2003a; Hendrickx *et al.* 2003b; Hendrickx & Maelfait 2003). For *P. piraticus*, genetic differentiation in life history traits was already established through quantitative genetic research (Hendrickx *et al.* 2008). Thus this species is

an ideal model to test to what extent selection between habitats can result in reproductive isolation.

Male and female *Pirata piraticus* Araneae: Lycosidae of two populations in Flanders Belgium were studied: (i) Damvallei (51°03' N, 3°50' E), an unpolluted freshwater marsh henceforth referred to as the reference population R; and (ii) Galgenschoor (51°18' N, 4°18' E,) a tidal marsh located along the river Scheldt, heavily polluted by nearby industrial activities, referred to as the polluted population P. These populations were selected based on earlier studies where they were shown to be two extremes of a life-history trait and pollution gradient (Hendrickx *et al.* 2003).

Picture 2 | View from Galgenschoor, Kallo, a metal polluted tidal marsh where Pirata piraticus spiders were collected.

5.3 Pardosa saltans

Picture 3 | Female Pardosa saltans with egg cocoon kept in the laboratory in a container lined with plaster and leaves.

Pardosa saltans (Töpfer-Hofmann *et al.* 2000) is a ground-dwelling wolf spider Lycosidae of open forests, forest fringes and clearings, dominated by *Fagus sylvatica, Quercus robur, Carpinus betulus* and *Anemone nemorosa* and is widespread across Western Europe (Edgar 1970; De Bakker *et al.* 2002; Töpfer-Hofmann *et al.* 2000; Hendrickx *et al.* 2001). In Belgium, the species occurs in both polluted and unpolluted habitats in the south and northwest (Hendrickx *et al.* 2001). They do not only inhabit wood fringes, but also recently felled woods (Hendrickx *et al.* 2001) and are dominant after wood fires (Moretti 2002). Like other Lycosid species, *P. saltans* forages on ground-dwelling prey, and the species may attain high local densities (Edgar 1970; Hendrickx *et al.* 2001), making their study and collection relatively easy.

It is closely related to *Pardosa lugubris*, from which it was taxonomically separated only recently, based on male courtship display behavior and small differences in the palpal cymbium (Töpfer-Hofmann *et al.* 2000). The species has been studied in great detail (Wilczek *et al.* 2004; Hendrickx *et al.* 2001; Framenau & Elgar 2005; Rickers *et al.* 2006; Edgar 1972; Segers 1989; Wilczek 2005; Nyffeler & Benz 1981; Wilczek *et al.* 2003; Babczynska & Migula 2002), but because of its recently changed taxonomic status, it is often unclear if this refers to *P. saltans* or *P. lugubris s.s.* In an earlier study on *P. lugubris* , Wilczek *et al.* (2003 and 2005) showed no significant changes in cell-level energetics and a larger increase in heat shock proteins in previously exposed populations when more stress was applied and higher acetylcholinesterase and carboxylesterase activity were present in metal-polluted habitats. This suggests efficient repair mechanisms protecting homeostasis and a well-developed capacity to detoxify.

P. saltans males show a very pronounced courtship display, which is described in detail by Töpfer-Hofmann *et al.* (2000). It can therefore be expected to be a more important cue for mate choice compared to *P. piraticus*, where courtship behavior is almost absent. Copulation durations were observed to be very long (F. Hendrickx, personal communication) and since females are carrying the males, this could induce substantial costs to the female because of increased visibility and energy consumption.

Study populations were located in the province of Liège in the southeast of Belgium. The region is known for the presence of natural metal-rich outcrops of zinc and lead that were industrially extracted since the 13th century. Extraction peaked during the 19th century and ended in 1970 (Duvigneaud and Jortay, 1987). Polluted sites were initially located based on a study on the distribution of *Viola calaminaria* Gingins, a typical metalliferous plant species that grows on soils with average soil metal concentrations of 11.886 mg/kg Zn, 24.3 mg/kg Cd and 9.342 mg/kg Pb (Bizoux *et al.* 2004). Three areas with high densities of *P. saltans* were selected as study sites. All three polluted sites were vegetated with plant species adapted to high soil metal concentrations calamine flora with *Silene vulgaris subsp. vulgaris var. humilis*, *Viola calaminaria*, *Thlaspi caerulescens subsp. calaminare*, *Armeria maritima subsp. halleri* and *Festuca ovina subsp. ophiolotique* as dominant species (Bizoux *et al.* 2004; Graitson & Goffart 2005; Cappuyns *et al.* 2006). La Rochette à Prayon Pond (CH-P1, 50°35'N, 5°40'E) and Bois les Dames (CH-P2 , 50°35'N, 5°39'E), both located in Chaudfontaine, are located at the east bank of the Vesder river. The metal enrichment originates from atmospheric pollution with metal dust (Graitson & Goffart 2005). Schmalgraf, La Calamine (CM-P, 50°42'N, 6°00'E) is a very small 1.5 ha site in the Hohnbach alluvial plain, close to the river Gueule, polluted through mining activities in nearby sites with natural ore deposits (Duvigneaud et al., 1979). All three sites are sparsely vegetated with a metal-adapted calamine flora (Bizoux *et al.* 2004; Graitson & Goffart 2005). Within these sites, *P. saltans* preferred patches of isolated *Quercus robur* trees. For each of these historically polluted sites, a nearby non-polluted reference site was selected consisting of recent clearings that were dominated by beech *Fagus sylvatica* and lacked a metalliferous flora: Louveigné (CH-R1 , 50°33'N, 5°42'E) and Fraipont (CH-

R2, 50°33'N, 5°44'E), both in Chaudfontaine, and Kelmis, La Calamine (CM-R, 50°42'N, 6°01'E). The study populations thus occur in a mosaic of both historically contaminated sites exposed to a mixture of metals as Cd, Zn, Pb and Cu and unexposed populations. Distances between the study populations within the municipalities of Chaudfontaine and La Calamine ranged between 1 and 4 km, while both municipalities were circa 20 km apart. Since sample areas were small 100 m² maximum and interspersed with unsuitable habitat for our study species i.e. grasslands, arable fields and urban areas, we expect gene flow among the populations to be partially reduced.

Picture 4 | Field collection of Pardosa saltans in La Rochette à Prayon Pond, Chaudfontaine.

Map 1 | Location of the 6 study populations of Pardosa saltans in the Southeast of Belgium and the 2 study populations on Pirata piraticus in the North of Belgium.

Map 2| Detail of the ocation of the 6 study populations of Pardosa saltans in the Southeast of Belgium.

6 GENERAL OBJECTIVES AND THESIS OUTLINE

The objectives of this research can be divided in three main research topics:

(i) Based on life history theory and known physiological metal defense mechanisms, we first evaluate whether *Pardosa saltans* populations exposed to metals
   a. Show evidence of reduced survival and growth rates, delayed timing of reproduction, lower reproductive investment, and/or the production of larger offspring;
   b. Show evidence of increased physiological defense based on MTLP production
   c. Show evidence of trade-offs among different life-history traits and of local adaptation to metal stress.

   To test these objectives, we combined a field study (chapter 1) with a laboratory experiment (chapter 2).

(ii) Second, we study the impact of metal pollution on population genetic parameters in *Pardosa saltans* using AFLP markers (chapter 3). We thereby predict:
   a. Lower genetic variation in populations exposed to metals;
   b. Stronger genetic differentiation between metal-exposed and reference populations compared to levels of differentiation within each group;
   c. The presence of a number of loci that can be linked to metal exposure (outlier loci).

   And we evaluate whether the populations can be considered locally adapted based on gentic evidence based on AFLP markers.

(iii) Third, we test whether metal pollution may affect mate choice through the by-product mechanism (chapter 4), and as such, cause reproductive isolation in *Pirata piraticus*. We thereby predict:
   a. Differential effects of metal stress on mating propensity, with naturally stressed spiders expected to be least affected;
   b. Assortative mating resulting from female preference to mate with males from their own population

(iv) And we evaluate whether based on these findings, the populations can be considered locally adapted.

Picture 5 | Reference study site Fraipont where Pardosa saltans was collected.

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Evolutionary biologists
ask questions inspired
by comparisons
of differences,
differences between species,
between populations,
between individuals.

They want to understand
why things are different,
not why they are the same

Stearns 2000

CHAPTER 1 | DIRECT AND INDIRECT EFFECTS OF METAL STRESS ON PHYSIOLOGY AND LIFE HISTORY VARIATION IN FIELD POPULATIONS OF A LYCOSID SPIDER

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ABSTRACT

1. Under stress, life history theory predicts reduced growth rates and adult sizes, reduced reproductive allocation, production of larger offspring and postponed reproduction. Both direct and indirect effects of metals can explain these trends, mainly linked to energetic constraints. Metallothionein-like proteins (MTLP's) are believed to be an important defense mechanism against the adverse effects of metals and other stressors.

2. We tested these predictions comparing six field populations of the wolf spider *Pardosa saltans*, three of which were on sites that are historically polluted with heavy metals.

3. As expected for life histories evolving under energetic constraints, adult size and condition correlated negatively and egg mass positively with Cd concentrations for a subset of four populations. In the population that showed the highest cadmium and zinc body burdens, reproductive output and allocation were lowest and reproduction was postponed.

4. Contrary to our expectation, for all six study populations MTLP concentrations did not increase in exposed populations, indicating that this defense mechanism cannot explain the observed variation in life histories.

5. We conclude that indirect and synergistic effects of metal pollution may be more important than physiological defense mechanisms in shaping life history traits in field populations.

Keywords: *Pardosa saltans*, metal, metallothionein, life history, ecological effects, field study

1 INTRODUCTION

Even in (semi-)natural habitats, organisms are often exposed to variable concentrations of pollutants that primarily originate from human activities. Among these, metals comprise one of the most persistent components, with strong direct and indirect impacts on both plant and animal populations (Hunter et al., 1987a, b; MacNair, 1998; Klerks and Levinton, 1993; Posthuma and Van Straalen, 1993; Das et al., 1997; Shaw 1999; Bertin and Averbeck 2006; Morgan et al., 2007). Direct physiological effects may occur when metals bind with essential molecules and alter their structure or function, which can lead to the inhibition of various enzymatic pathways and anti-oxidant activity. Indirect physiological effects may occur when reactive oxygen species are produced that disturb lipid structure, cause lipid peroxidation or apoptosis (Viarengo and Nott, 1993; Lukkari et al., 2004; Wilczek, 2005; Bertin and Averbeck, 2006). While less well understood, metal pollution may also impact natural populations through cascading ecological effects. For instance, pollution-driven changes in vegetation structure or reduction in prey availability may result in a reduction in fitness due to increased intra- and interspecific competition for critical resources and changes in predation risk, or in a reduction in reproductive output or survival due to a lower energy budget (Van Hook and Yates, 1975; Hunter et al., 1987b; Posthuma and Van Straalen, 1993; Lock et al., 2003). Population-level changes may, in turn, cause effects at community level, especially if highly sensitive species are keystone members, and may result in alterations in critical ecosystem functions (Coughtrey et al., 1979; Hunter et al., 1987a; Tyler et al., 1989; Read et al., 1998; Nahmani and Lavelle, 2002; Creamer et al., 2008; Clements and Rohr, 2009).

However, organisms dispose of a variety of defense mechanisms against adverse effects of metals, such as intake avoidance, decreased accumulation, increased excretion, detoxification or sequestration in a non-toxic form (Bengtsson et al., 1992; Posthuma and Van Straalen, 1993; Viarengo and Nott, 1993; Wilczek et al., 2004; Xie and Klerks, 2004; Bahrndorff et al., 2006; Morgan et al., 2007). Metallothionein-like proteins (MTLPs) are believed to play an important physiological role in defense

against metal stress. MTLPs are non-enzymatic proteins with a low molecular mass and high affinity for metals, mainly Cu, Zn and Cd, due to their high cysteine content consisting of sulphydryl-groups that directly bind metals in insoluble fractions (Viarengo and Nott, 1993; Dallinger, 1996; Roesijadi, 1996; Nordberg, 1998; Park et al., 2001; Amiard et al., 2006; Janssens et al., 2009). While MTLPs are constitutively expressed, their synthesis can also be induced upon exposure to metals, which may render them suitable biomarkers for metal exposure (Hopkin, 1989; Dallinger and Rainbow, 1993; Viarengo and Nott, 1993; Roesijadi, 1996; Viarengo et al., 1999). Induction levels have been shown to vary among individuals, species, metals, and biotic and abiotic conditions (Amiard et al., 2006).

Correlative as well as experimental studies on invertebrate populations from polluted sites have revealed multiple and often complex effects of metals on life-history traits such as development time, survival, body size, age at reproduction, fecundity and egg size (Bengtsson et al., 1992; Posthuma and Van Straalen, 1993; Posthuma et al., 1993; Shirley and Sibly, 1999; Hendrickx et al., 2003b; Lagisz and Laskowski, 2008). Metal-induced changes in life-history traits can be considered adaptive if they result in fitness increases in individuals originating from polluted sites relative to individuals from non polluted sites ('life history adaptation' *sensu* Posthuma and Van Straalen, 1993; Klerks and Weis, 1987; Brandon, 1991; Bengtsson et al., 1992; Mouneyrac et al., 2002; Kawecki and Ebert, 2004). However, life-history changes may also reflect non-adaptive trade-offs with traits associated with tolerance mechanisms against pollution. Because these mechanisms are believed to be energetically costly, life history theory predicts resource demanding traits, such as growth and fecundity, to be reduced under metal stress (Southwood, 1988; Sibly and Calow, 1989; Calow, 1991; Donker et al., 1993a; Posthuma et al., 1993; Zera and Harshman, 2001). Body condition (i.e. body mass relative to body size) is considered a sensitive individual-based measure of stress effects (Danielson-François et al., 2002; Aisenberg, 2009). While body size is fixed after maturation (Hagstrum, 1971; Hendrickx and Maelfait, 2003), body mass varies with nutritional status and energetic balance and is often positively correlated with fecundity (Jones and Hopkin, 1998; Danielson-

François et al., 2002). When energetically stressed, females can be expected to invest in less but larger offspring relative to their own size since the latter generally mature earlier, develop faster, and survive better (Van Noordwijk and deJong, 1986; Stearns, 1992; Fox and Csezak, 2000; Tamate and Maekawa, 2000; Hendrickx et al., 2003b). In metal-exposed populations, individuals can also be expected to mature earlier to counter the adverse effects of metals that accumulate during lifetime (Sibly and Calow, 1986, 1989; Posthuma and Van Straalen, 1993; Tranvik et al., 1993; Donker et al., 1993 a,b). However, energetic constraints in polluted environments may also cause non-adaptive delays in reproduction (Colvin and Gatehouse, 1993; Winterer and Weis, 2004). Such stress-induced shifts in reproductive timing in response to stress have been widely studied in plants but less so in animals, despite their presumed effects on population dynamics and, together with changes in body size, on reproductive isolation between stressed and reference populations (Fox, 2003; Winterer and Weiss, 2004; Weis et al., 2005; Eraly et al. 2009).

Here we quantify effects of metal stress on MTLP concentrations and life-history traits in a ground-dwelling lycosid spider *Pardosa saltans* (Töpfer-Hofmann et al., 2000). Spiders play a key role in ecosystem functioning and constitute macro-concentrators of metals as predators and polyphages (Dallinger and Rainbow, 1993; Maelfait and Hendrickx, 1998; Heikens et al., 2001; Tojal et al., 2002; Hendrickx et al., 2003a; Jung et al., 2008b). As opposed to insects, which mainly excrete metals (Van Straalen et al., 1987; Janssen et al., 1991; Lindqvist and Block, 1994, 1995; Kramarz, 1999), detoxification in spiders is predominantly based on storage of metals in intracellular granules (Breymeyer and Odum, 1969; Van Hook and Yates, 1975; Brown, 1982; Hopkin and Martin, 1985; Janssen et al., 1991; Kramarz, 2000; Hendrickx et al., 2003a). Higher concentrations of metals are measured in the midgut, or hepatopancreas, that has a low metabolic rate and may serve as a protective barrier for other sensitive organs such as the gonads (Ludwig and Alberti, 1988; Hopkin, 1989; Posthuma and Van Straalen, 1993; Foelix, 1996; Wilczek and Babczynska, 2000). While spiders have higher internal metal concentrations compared to most other soil-dwelling invertebrates (e.g. Van Hook and Yates, 1975; Hunter et al., 1987a; Larsen et al., 1994; Rabitsch, 1995; Maelfait, 1996; Wilczek and

Migula, 1996; Maelfait and Hendrickx, 1998; Stone et al., 2001), they do show considerable between-species variation related to lifestyle. For instance, lycosid spiders generally have higher Cd, Zn, Pb and Cu burdens than web-building spiders, likely as a result of their more direct contact with polluted soil (Van Hook and Yates, 1975; Nyffeler and Benz, 1981; Salo et al., 1991; Marczyk et al., 1993; Larsen et al., 1994; Wilczek and Migula, 1996; Hendrickx et al., 2004; Wilczek et al., 2008; Jung et al., 2008a).

Here we assess if, and to what extent, life history and physiological traits of *P. saltans* are affected by metal pollution under field conditions. Contrary to most laboratory experiments, indirect effects of metal pollution are also taken into account. We tested the following predictions: (i) internal concentrations of Zn, Cd and Cu and MTLP are higher in individuals from polluted than from reference sites; (ii) individuals from polluted sites have smaller adult body sizes and lower body conditions, and produce fewer but larger offspring than individuals from reference sites; and (iii) individuals from polluted sites are expected to differ in maturation time from individuals from reference sites.

2 MATERIAL AND METHODS

2.1 Study species

Pardosa saltans is a lycosid spider of open forests, forest fringes and clearings, and is widespread across Western Europe (Edgar, 1970; Alderweirt and Maelfait, 1990; De Bakker et al., 2000; Roberts, 1998; Töpfer-Hofmann et al., 2000; Hendrickx et al., 2001). In Belgium, the species occurs in both polluted and unpolluted habitats in the south and northwest (Hendrickx et al., 2001). It is closely related to P. lugubris, from which it was taxonomically separated only recently, together with other species of the Pardosa lugubris s.l. complex, based on male courtship display behavior (Töpfer-Hofmann et al., 2000). Like other lycosid species, P. saltans forages on ground-dwelling prey, and the species may attain high local densities (Edgar, 1970; Hendrickx et al., 2001). Because females

carry their eggs in a cocoon attached to their spinnerets (Roberts, 1998), female reproductive output (cocoon mass, number of eggs, and egg mass) can accurately be assessed and related to female characteristics.

2.2 Field sampling

Study populations were located in the province of Liège (southeast Belgium). The region is known for the presence of natural metal-rich outcrops of zinc and lead that were industrially extracted since the 13th century. Extraction peaked during the 19th century and ended in 1970 (Duvigneaud and Jortay, 1987). Polluted sites were initially located based on a study on the distribution of *Viola calaminaria* (Gingins), a typical metalliferous plant species that grows on soils with average soil metal concentrations of 11.886mg/kg Zn, 24.3 mg/kg Cd and 9.342 mg/kg Pb (Bizoux et al., 2004). Three areas with high densities of *P. saltans* were selected as study sites. La Rochette à Prayon Pond (CH-P1, 50°35'N, 5°40'E) and Bois les Dames (CH-P2 , 50°35'N, 5°39'E), both in Chaudfontaine, are located at the east bank of the Vesder river and contaminated due to atmospheric pollution with metal dust (Graitson et al., 2005). Schmalgraf, La Calamine (CM-P, 50°42'N, 6°00'E) is a very small (1.5 ha) site in the Hohnbach alluvial plain, close to the river Gueule, polluted through mining activities in nearby sites with natural ore deposits (Duvigneaud et al., 1979). All three sites are sparsely vegetated with a metal-adapted calamine flora (Bizoux et al., 2004; Graitson et al., 2005). Within these sites, P. saltans preferred patches of isolated *Quercus robur* trees. For each of these historically polluted sites, a nearby non-polluted (reference) site was selected consisting of recent clearings that were dominated by beech and lacked a metalliferous flora: Louveigné (CH-R1 , 50°33'N, 5°42'E) and Fraipont (CH-R2, 50°33'N, 5°44'E), both in Chaudfontaine, and Kelmis, La Calamine (CM-R, 50°42'N, 6°01'E). Distances between the study populations within the municipalities of Chaudfontaine and La Calamine ranged between 1 and 4 km, while both municipalities were circa 20 km apart. Since sample areas were small (100 m² maximum) and interspersed with unsuitable habitat for our study species (i.e. grasslands, arable fields and urban areas), we expect gene flow among the

populations to be partially reduced. However, few studies showed that even under high levels of gene flow, genetic divergence in ecologically relevant traits is possible (Dhuyvetter et al. 2007).

Starting on April 3rd 2007, individuals were counted, sexed and aged (by developmental stage) on a weekly base during a period of 8 weeks in sites CH-R1 (51-210 per period), CH-R2 (30-102 individuals per period), CH-P1 (60-72 per period) and CH-P2 (49-92 per period). Due to the large amount of work to monitor these populations on a weekly basis, only four out of six populations could be used for life history assessments. Adult males and females with egg cocoons were randomly collected alive by hand picking and transported individually in plastic containers to prevent cannibalism and avoid mixing of cocoons and mothers. Upon arrival in the lab, individuals were frozen alive at -20 °C for subsequent measurements. In 2008, ten females carrying a cocoon were sampled at random in all six populations to determine metal body burdens and MTLP concentrations. We restricted our analyses to females because former studies showed differences in metal burdens between sexes (Wilczek et al., 2004, 2008).

2.3 Metal and Metallothionein-like protein (MTLP) analyses

Estimating the degree of metal exposure of each population was based on the metal body burdens of the spiders rather than soil concentrations as physicochemical properties of the soil and prey composition hamper the translation of soil concentrations to exposure levels (Dallinger et al., 1992; Hopkin 1993; Maelfait, 1996; Van Straalen et al., 2001 Du Laing et al 2002; Tojal et al 2002; Hendrickx et al. 2004). Moreover, as the excretion of ingested metals in spiders is virtually absent, their internal metal concentration is the most accurate reflection of their lifetime exposure (Hendrickx et al. 2003).

After recording the fresh weight (Mettler Toledo, AT261, Deltarange), we added 350 µl of buffer A (10 mM tris HCl and 85 mM NaCL, pH 7.4, Sigma-Aldrich, USA) and homogenized each individual during one minute on ice in new polypropylene vials with a Tissue Ruptor (Qiagen). After extracting

100 µl homogenate for MTLP measurement, samples were retained for measuring the metal concentration. To determine internal metal burdens, the remainder of the sample was first dried at 60°C for 48 hours, and a nitric acid (70%, Merck, Pro Analysis, Germany)/hydrogen peroxide (30%, Merck, Pro Analysis) microwave digestion (Blust et al., 1988) was performed. All samples were diluted with Milli-Q water (Millipore, USA) and weighed again. Cd, Zn and Cu concentrations were measured using ICP-MS (Varian Ultra Mass 700, Australia). Certified mussel reference material (CRM 278) of the EU Community Bureau of Reference was used as a quality control. Recoveries were within 10% of the certified values.

To measure MTLP concentrations, 100 µl samples were centrifuged (20 min, 13200 g, 4 °C; Eppendorf Centrifuge 5804R, Germany), 50 µl of the supernatans was separated and stored at -80°C until analyzed. A cadmium saturation thiomolybdate assay (Klein *et al.*, 1994) was applied. During this process, oxidized MTLP is converted to native MTLP by 2-mercaptoethanol and a metal donor Zn^{2+}, followed by saturation with the radioactive Cd^{109} isotope. Cd^{109}- concentrations were then quantified with a Minaxi-Autogamma 5530 counter (Canberra Packard, USA). To calculate Cd-MTLP concentrations (expressed as nmol/g wet weight), we assumed total saturation of MTLP with the metal ions at a ratio of 7 mol Cd per mol MTLP, as has been demonstrated for vertebrates and most invertebrates (Kito et al., 1982; Viarengo and Nott, 1993; Hensbergen et al., 2000; Dabrio et al., 2002). Metal concentrations were both expressed in µg (for comparison with other studies) and nmol per g wet weight (better insight in biological effects and for comparison with MTLP concentrations).

To assess the extent to which Cd-, Zn- and Cu-molecules were bound by MTLPs, we also estimated the concentration of free metals by assuming a metal stoichiometry of 7 mol Cd, 12 mol Cu and 7 mol Zn per mol MTLP (Kito et al., 1982; Viarengo and Nott, 1993; Hensbergen et al., 2000; Hollis et al., 2001; Dabrio et al., 2002). Since MTLP's have the highest affinity for Cd, which is the most toxic metal, we also calculated this measure for Cd alone.

2.4 Life history traits

Adult males and females (restricted to females with cocoon) their mass was measured in frozen state to the nearest 0.01 mg on a Galaxy™ 110 Ohaus balance after which individuals were stored in a 100% ethanol solution (Merck, Pro Analysis, Germany). Maximum cephalothorax width (Hagstrum, 1971) of all adult spiders on ethanol was measured with a Wild M3 stereomicroscope with eyepiece graticule (Heerburg, Switserland; measurement error = 0.06 mm; male and female cephalothorax width). Body condition was determined by regressing spider mass against cephalothorax width. To quantify reproductive output and investment, we measured cocoon mass in frozen state, reproductive allocation by regressing cocoon mass against cephalothorax width, fecundity expressed as the number of eggs or young within the cocoon, and average egg mass, determined as the weight of the cocoon divided by the number of eggs. The weight of the silk surrounding the cocoon was not detectable by our balance (<0.01 mg) and does therefore not affect our estimates of cocoon weight and egg size. To study phenological differences between the four CH study populations, we compared the relative proportions of subadult spiders, adult males and females, and females with cocoon, captured during a period of 8 weeks (spring 2007).

2.5 Statistical analysis

We applied general linear models and post-hoc Tukey tests to compare metal burdens, MTLP-concentrations and life history traits among populations (proc glm, SAS 9.1, SAS Institute Inc ©). To model co-variation between female size and values of the reproductive traits, female cephalothorax width was included as co-variable. To scale the dimensions of the various traits, cube root values of body mass were used. To model trade-offs between egg mass and fecundity, fecundity estimates were regressed against egg mass (cube rooted), while including female cephalothorax width as a covariate. When residuals did not fulfill the assumptions of normality (Shapiro-Wilk test statistic) and homoscedasticity (Levene's test), dependent variables were $\log_e$-transformed.

To analyze the significance and direction of the relationships between Cd concentration and life history traits, ordered heterogeneity tests were performed per trait (Rice and Gaines, 1994). The statistic of this test constitutes the product r_sP_c, with r_s being the Spearman rank correlation coefficient of average metal concentration against average life history trait value per population, and P_c the complement of the p-value of the respective factor testing variation in life history traits among populations (ANOVA).

We used general linear models to test for relationships between MTLP concentrations and Cd, Cu and Zn concentrations (both in nmol/g ww) and to compare the direction and strength of these relationships among populations. Estimated free metal concentrations were compared between populations with an Anova test with post-hoc Tukey comparison (proc glm, SAS 9.1, SAS Institute Inc ©). We used polynomial models to compare non-linear temporal trends in proportion of adult males, adult females, and females with cocoon among populations by including a quadratic factor (period*period) and the two-factor interaction with population (proc genmod with binomial distribution, SAS 9.1, SAS Institute Inc ©). Error variance components were corrected for overdispersion.

3 RESULTS

3.1 Metal body burden

Internal Cd-burdens significantly differed among populations ($F_{5,57} = 47.54$; $P < 0.0001$), with the highest values measured in CH-P1 and CH-P2. Populations CM-P, CH-R1 and CH-R2 did not mutually differ in mean Cd-burdens but the latter two showed significantly higher values than CM-R and lower ones than CH-P2 and CH-P1 (Fig. 1A). Zn-burdens also significantly differed among populations ($F_{5,57} = 4.55$, $P = 0.0016$), with lower values in CH-R1 compared to the three polluted sites (Fig. 1B). Cu-concentrations also significantly differed among populations ($F_{5,57} = 13.27$; $P < 0.0001$), however, the overall pattern was inversed compared to Cd and Zn, i.e. values in CH-R1 were significantly higher compared to the three

polluted populations and CH-R2, while values in CM-P were lower than in CH-R2 and CM-R and values in CM-R were higher than in CH-P2 (Fig. 1C).

3.2 MTLP concentrations

MTLP concentrations did not significantly differ among the studied populations ($F_{5,57}$ = 1.31, P = 0.27; Fig. 2). However, estimated concentrations of free metals were always larger than zero and differed significantly among populations ($F_{5,57}$ = 3.32, P = 0.011), with the highest concentration in CH-P1 and CH-P2 and the lowest concentration in CH-R1. When only considering Cd-binding potential, among-population differences were even stronger ($F_{5,57}$ = 31.74, P < 0.0001), with higher concentrations in CH-P1 and CH-P2 than in CH-R1, CH-R2, CM-R and CM-P (all without free Cd molecules. Fig. 2). MTLP concentrations were not significantly correlated with internal body burdens of Cd, Cu or Zn, neither for the entire dataset, nor within populations (all P between 0.15- 0.95).

Picture 1/ Male Pardosa saltans spiders captured and marked in reference population Louveigné.

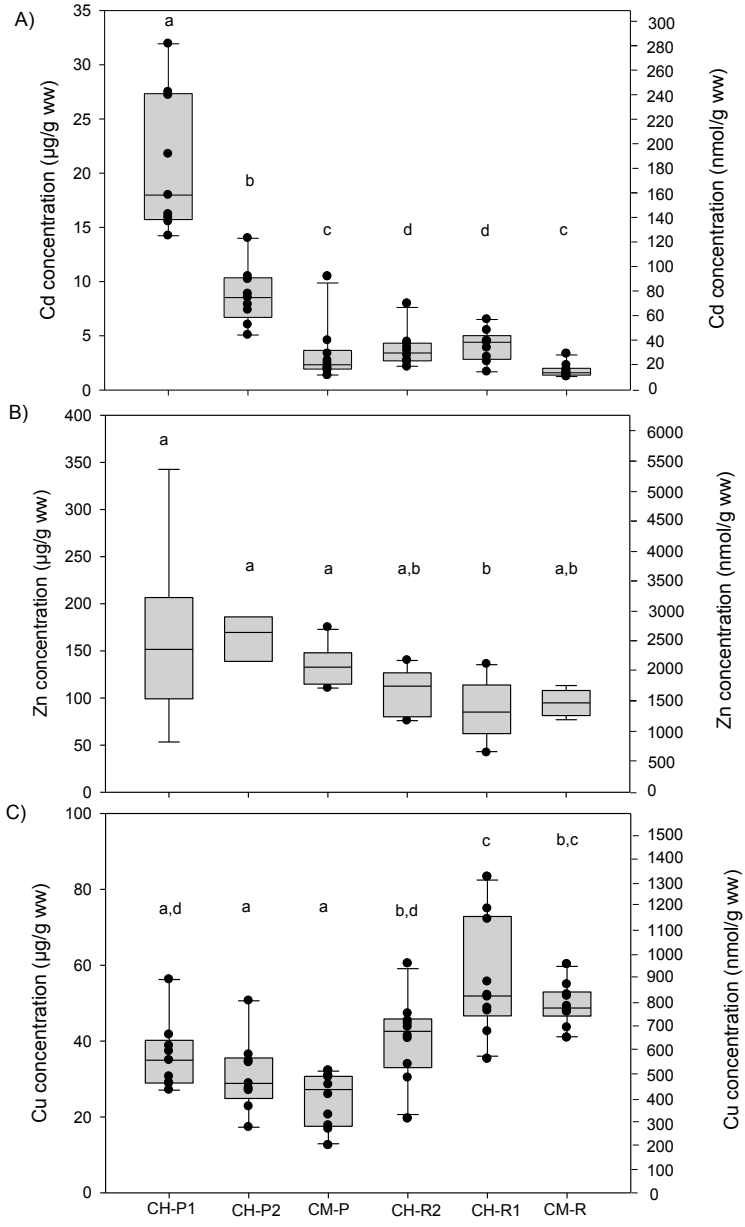

Figure 1 | Boxplot diagrams of Cd (A), Zn (B) and Cu (C) concentrations (expressed both as µg and nmol per g wet weight) in three polluted (-P) and three reference (-R) populations of P. saltans. Horizontal lines represent average values; boxes represent upper and lower quartiles, whiskers represent 90th and 10th percentiles; dots represent extreme values. Populations with the same superscript letters do not significantly differ.

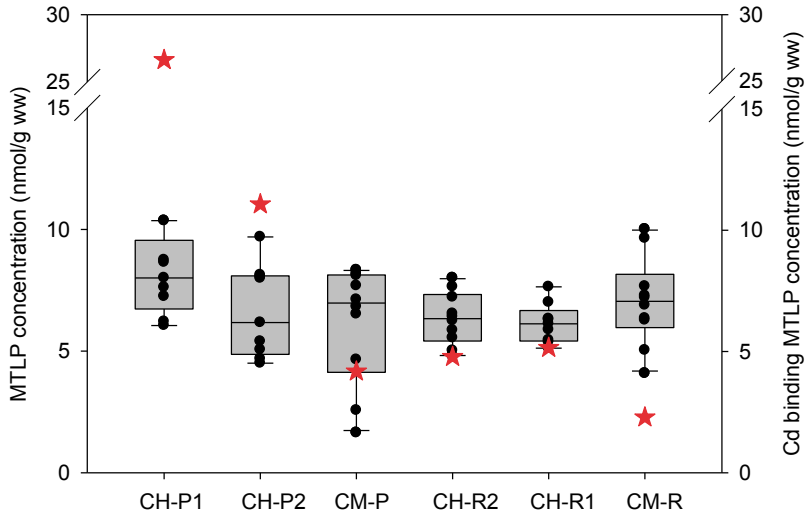

Figure 2 | Boxplot diagram of MTLP concentrations in three polluted (-P) and three reference (-R) populations of P. saltans. Horizontal lines represent average values; boxes represent upper and lower quartiles, whiskers represent 90· and 10· percentiles; dots represent extreme values. Stars indicate average MTLP concentrations required for binding all Cd.

3.3 Life history traits

Male cephalothorax width, body mass and condition significantly differed among populations (cephalothorax width: $F_{3,158} = 21.77$; $P < 0.0001$; body mass: $F_{3,145} = 7.3$; $P = 0.0001$; Fig. 3 A,B; condition: $F_{3,145} = 3.03$; $P = 0.032$) and were negatively correlated with Cd-burdens (cephalothorax width: $r_sP_c = -0.8$; $P = 0.01$; body mass: $r_sP_c = -0.6$; $P = 0.05$). Female cephalothorax width, body mass and condition significantly differed among populations (cephalothorax width: $F_{3,205} = 17.6$; $P < 0.0001$; body mass: $F_{3,228} = 26.84$; $P < 0.0001$; Fig. 3 C,D; condition: $F_{3,205} = 6.11$; $P = 0.0005$). At population level, Cd burden was inversely related to female mass ($r_sP_c = -0.8$; $P = 0.01$), but not to cephalothorax width ($r_sP_c = -0.4$; $P = 0.1$) although females from the most polluted (CH-P1) and least polluted (CH-R1) population were smallest and largest, respectively. Body condition of CH-R1 females was significantly larger than of CH-P1 and CH-P2 females.

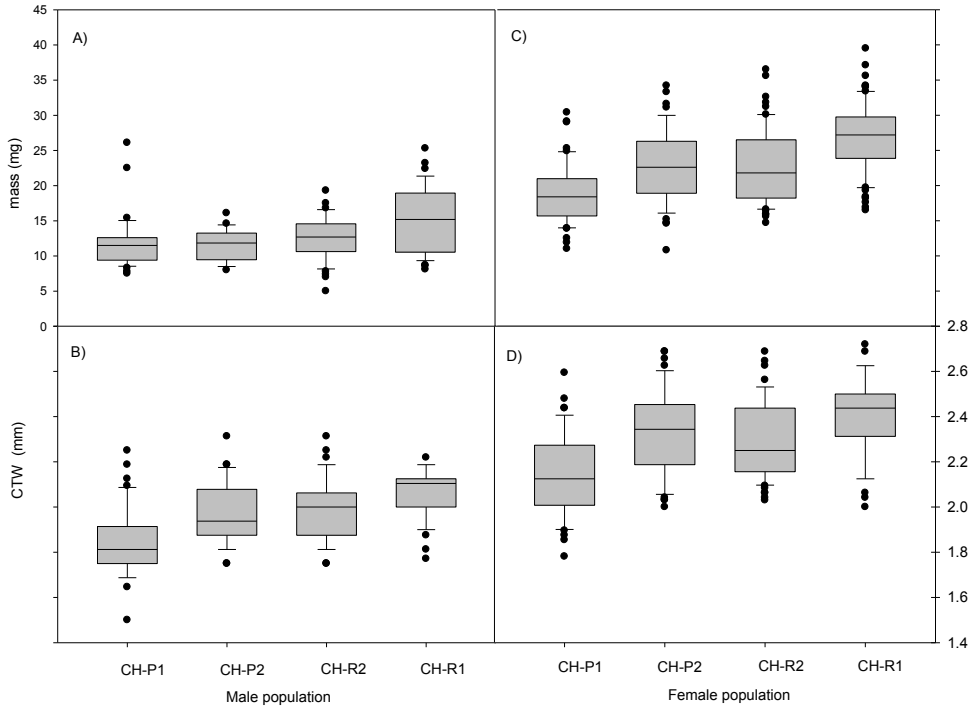

Figure 3 | Boxplot diagrams of male and female body mass (A, C), and cephalothorax width (CTW; B, D) in two polluted (-P) and two reference (-R) populations of P. saltans. Horizontal lines represent average values; boxes represent upper and lower quartiles, whiskers represent 90th and 10th percentiles; dots represent extreme values.

Cocoon mass significantly differed among the studied populations ($F_{3,227}$ = 30.99, P < 0.0001; Fig. 4A) but was not significantly correlated with Cd-burden (r_sP_c = -0.4; P = 0.1). Cocoon mass was lowest in CH-P1, intermediate in CH-R2 and highest in CH-P2 and CH-R1. Cocoon mass increased with increasing cephalothorax width (slope = 0.81; $F_{1,204}$ = 127.48, P < 0.0001) and this relationship did not significantly differ among populations ($F_{3,204}$ = 1.44; P = 0.23). Reproductive allocation also differed among populations ($F_{3,204}$ = 12.12; P < 0.0001) and was highest in CH-P2 and CH-R1 and lowest in CH-P1 and CH-R1.

The number of eggs in a cocoon significantly differed among populations ($F_{3,202}$ = 29.14, P < 0.0001; Fig. 4B) but was not significantly correlated with Cd burden (r_sP_c = -0.4; P = 0.1). Females of CH-R1 and CH-P2 produced

significantly more eggs than those of CH-R2, while females from the most heavily polluted population (CH-P1) produced significantly less eggs than those from CH-R1, CH-P2 and CH-R1. The fecundity was positively correlated with cephalothorax width (b = 29.11; $F_{1,179}$ = 100, P < 0.0001), and this relationship did not differ significantly among populations ($F_{3,179}$ = 0.77, P = 0.51). When taking variation in female cephalothorax width into account, the difference in fecundity remained significant among populations ($F_{3,179}$ = 5.63, P = 0.0011) and females of the most heavily polluted population CH-P1 still produced a lower number than those from CH-R1, CH-P2 and CH-R2.

Egg mass significantly differed among populations ($F_{3,200}$ = 3.07, P = 0.029; fig. 4C), and females from populations with a higher average Cd-burden produced larger eggs (r_sP_c = 0.78; P = 0.015). Egg mass was not significantly correlated with maternal cephalothorax width ($F_{1,178}$ = 0.6, P = 0.44) and was negatively correlated with fecundity (b = -0.0026; $F_{1,177}$ = 36.31, P < 0.0001). This relationship did not differ among populations ($F_{3,177}$ = 2.42, P = 0.07).

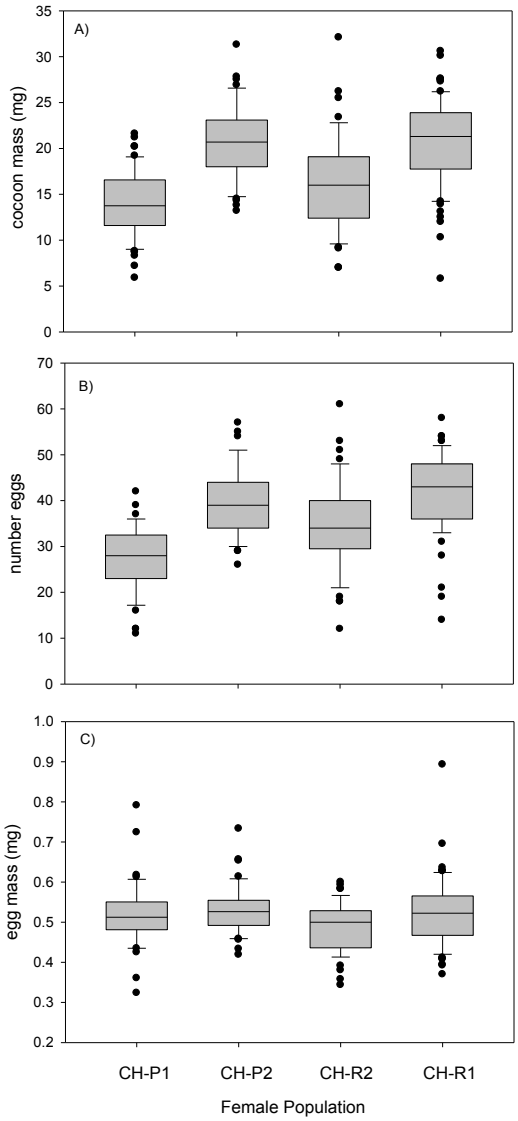

Figure 4 | Boxplot diagrams of cocoon mass (A), egg number (B) and egg mass (C) in two polluted (-P) and two reference (-R) populations of P. saltans. Horizontal lines represent average values; boxes represent upper and lower quartiles, whiskers represent 90th and 10th percentiles; dots represent extreme values.

3.4 Phenology

Adult males and females were present from the first and second week of April onwards, respectively. Females with cocoons were present from the end of April onwards, while all adult males had disappeared by the first week of June. Whereas temporal shifts in the proportion of adult males and females significantly differed among sites (Table 1; Fig. 5), none of the pairwise comparisons remained significant after correction for multiple testing. Females from the most polluted population (CH-P1) that carried a cocoon showed a slower increase in numbers with time and peaked later during the season compared to conspecifics from the least polluted population (CH-R1) (Figure 5).

	Proportion adult males		Proportion adult females		Proportion Females with cocoon	
		P		P		P
Site	2.53	0.47	3.37	0.34	**9.42**	**0.024**
Period	**19.8**	**<0.0001**	**39.85**	**<0.0001**	**71.71**	**<0.0001**
Period*period	**27.52**	**<0.0001**	**45.25**	**<0.0001**	**44.48**	**<0.0001**
Site*period	**8.03**	**0.046**	**14.05**	**0.0028**	**12.89**	**0.0049**
Site*period*period	3.73	0.29	0.57	0.9	**15.5**	**0.0014**

Table 1 | Generalized linear model testing temporal shifts in sex distribution in two polluted and two reference populations of P. saltans during an eight week sampling period. Significant effects are indicated in bold.

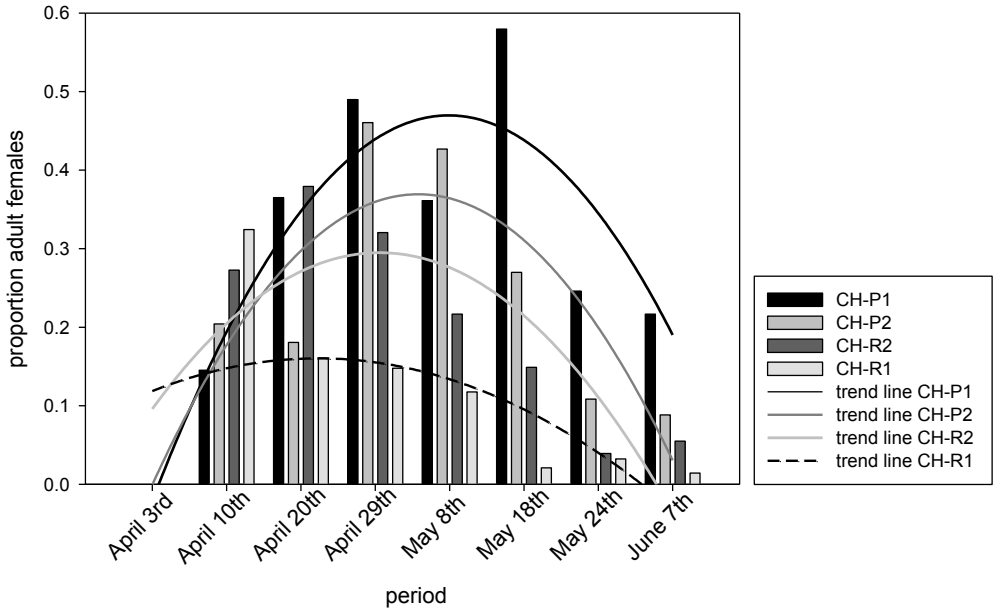

Figure 5 | Temporal shifts in adult female distribution in two polluted (-P) and two reference (-R) populations of P. saltans during an eight week sampling period. Trend lines represent the quadratic functions for each population.

4 DISCUSSION

As expected from life history theory, adult female *P. saltans* were smaller and in poorer body condition, delayed their reproductive period and produced heavier eggs under increased cadmium stress. Given the positive relationship between female size and reproductive output, cocoon masses and egg numbers were reduced in the most polluted sites. This decrease in fecundity was further increased by a lower relative amount of energy devoted to reproduction. In the least Cd and Zn polluted site, adult males and females were largest and females carried the heaviest cocoons and showed the highest fecundity. Populations from intermediately-polluted sites showed life history traits that resembled those from unpolluted reference sites. The observed shifts in life history traits could induce population differentiation, as for example a shift in reproductive period

(Winterer and Weis, 2004) and size assortative mating (Eraly et al., 2009), may ultimately induce reproductive isolation between populations.

Life history responses of exposed populations exhibited a rather large amount of variation and effects often were only present in the most polluted population, suggesting that metal concentrations in natural populations are to exceed threshold values before resulting in measurable effects. Life history alterations in response to metal pollution have been shown in other invertebrate groups such as lumbricids (Rozen, 2006), isopods (Donker et al., 1993a, b) and collembolans (Posthuma et al., 1993; Tranvik et al., 1993). However, reported directions of these changes were not consistent. For instance, isopods from polluted areas tended to mature earlier, rather than later, than in unpolluted sites and also showed higher, not lower, levels of reproductive output in the former (Donker, 1993a, b). Patterns opposite to those expected from life history theory can result from the fact that metal body burdens in species such as isopods affect their life histories only when they approach lethal concentrations after long term exposure. This induces higher mortality at later ages, which is expected to select for earlier maturation. For our model organism, Cd body burden in the most exposed population is still far below the lethal body concentration, estimated as 1050 mg Cd/kg DW for the congeneric species Pardosa astrigera (Jung et al., 2007).

Contrary to our expectations, MTLP concentrations were not significantly higher in populations from polluted sites, despite their higher cadmium and zinc concentrations. This implies larger concentrations of free, potentially toxic, metals in more polluted populations. Results of this study therefore do not support induced expression (acclimation) or increased constitutive production (adaptation) of MTLP. Nevertheless, peak Cd concentrations in our specimens were relatively high and in the same order of magnitude compared to other ecotoxicological studies in spiders, taken into account an average fresh to dry weight ratio of 4 in this species (Hunter, 1987b; Rabbitsch, 1995; Wilczek and Migula, 1996; Wilczek and Babczysnka, 2000; Heikens et al., 2001; Hendrickx et al., 2003a; Jung et al., 2007; Wilczek et al., 2004).

Lack of an increase in MTLP concentration under increased metal stress in this study contrasts with results obtained from an experimental study on the same populations that showed elevated MTLP concentrations when spiders were fed with cadmium spiked fruit flies (Eraly et al., 2010). Hence, while *P. saltans* appears physiologically able to induce increased MTLP expression in response to metal exposure, MTLP levels observed under polluted field conditions were comparable to those measured in individuals from unpolluted reference populations and from control laboratory treatments. It therefore seems reasonable to assume that field concentrations of metals are often too low to trigger increased MTLP concentrations, even though this implies free molecules of Cd and other metals. However, the role of MTLP's is broader than metal defense since they are also involved in (i) homeostasis of essential metals, (ii) non-toxic metal donorship to metalloproteins, (iii) antioxidant defense, and (iv) activities of gene regulatory compartments (Stegeman et al., 1992; Roesijadi, 1996; Amiard et al., 2006). Moreover, other parameters that differ between natural and laboratory conditions, such as temperature, hormones, food availability and pesticides may affect MTLP concentrations as well, thereby masking direct effects of metals (Viarengo et al., 1999; Wilczek, 2005; Amiard et al., 2006). Results from this and other studies (Roesijadi, 1996; Mouneyrac et al., 2002; Amiard et al., 2006; Forbes et al., 2006; Morgan et al., 2007; Santiago-Rivas et al., 2007) hence plead for a cautious use of MTLP as universal biomarker, as previsouly advocated by Hopkin (1989), Dallinger and Rainbow (1993) and Viarengo et al. (1999). Besides, other metal protection mechanisms, like storage in Metal Rich Granules (Mason and Jenkins, 1995), glutathione (Viarengo and Nott, 1993; Wilczek et al., 2008) and esterases (Wilczek, 2005) are known to be important in spiders and their concentrations differ depending on the degree of pollution. The detoxification pathway of MRGs' is not independent of MTLP's since evidence is present for lysosomal products of MTLP to breakdown remaining as insoluble residual bodies (Viarengo and Nott, 1993; Amiard et al., 2006).

Absence of measurable effects on individual growth and survival in a laboratory study (Eraly et al., 2010), despite increased MTLP production and internal Cd concentrations that were three times higher than those

measured in the most polluted field population (this study), suggests that indirect and/or synergistic effects of metal pollution may be more important in shaping life history variation in natural populations (Clements and Rohr, 2009). For instance, changes in prey species composition (Klerks, 2002), reduced prey availability or decreased energetic value of prey due to the toxicity of metals to less tolerant species (Clements and Rohr, 2009; Tranvik et al., 1993; Jung et al., 2008b) and changes in vegetation (Donker et al., 1993b; Posthuma et al., 1993; Graitson et al., 2005; Jung et al., 2008b) may all result in reduced resource availability. In support of this, most field studies reported a lower diversity of soil and ground-running macro-invertebrates on more polluted sites (Bengtsson and Rundgren, 1984; Read et al., 1998; Lock et al., 2003; Creamer, 2008). Discrepancies between laboratory and field studies, such as reported here, hence plead for rigorous and long term field testing in order to reveal population effects of toxicants (Lock et al., 2003; Amiard et al., 2006; Clements and Rohr, 2009).

Conclusion

From this study on 4 field populations of *Pardosa saltans* we can conclude life history and physiological traits of *P. saltans* are affected by metal pollution under field conditions, however not always in a straightforward manner: (i) internal concentrations of Zn and Cd are higher in individuals from sites containing a metalliferous flora than those without, except for CM-P; Cu concentration however showed a reversed trend; contrary to the expectations MTLP levels were not significantly higher in individuals from contaminated sites; (ii) males are lighter, smaller and have a lower body conditions when Cd concentrations are higher, while in females only body mass is negatively influenced. Reproductive values (cocoon mass, fecundity and egg mass) only were affected in the population with highest internal Cd (CH-P1); and (iii) the number of females carrying a cocoon peaked later in the season in the most polluted site.

Author's contribution

Debbie Eraly and Frederik Hendrickx conceived and designed the study and wrote the manuscript together with Luc Lens. Debbie Eraly prepared the samples for analysis under supervision of Lieven Bervoets. Debbie Eraly and Frederik Hendrickx analyzed the data. Thierry Backeljau provided usefull feedback on the manuscript.

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Whatever
does not kill me
makes me
stronger

Friedrich Nietzsche, 1888,
Twilight of the idols

CHAPTER 2 | EXPERIMENTAL EXPOSURE TO CADMIUM AFFECTS METALLOTHIONEIN-LIKE PROTEIN LEVELS BUT NOT SURVIVAL AND GROWTH IN WOLF SPIDERS FROM POLLUTED AND REFERENCE POPULATIONS

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ABSTRACT

Both local adaptation and acclimation in tolerance mechanisms may allow populations to persist under metal pollution. However, both mechanisms are presumed to incur (energetic) costs and to trade-off with other life history traits. To test this hypothesis, we exposed *Pardosa saltans* (Lycosidae) spiderlings originating from metal-polluted and unpolluted sites to a controlled cadmium (Cd) treatment, and compared contents of metal-binding metallothionein-like proteins (MTLPs), internal metal concentrations, and individual survival and growth rates with a reference treatment. While increased MTLP concentrations in offspring originating from both polluted and unpolluted populations upon exposure indicates a plastic tolerance mechanism, survival and growth rates remain largely unaffected, independent of the population of origin. However, MTLP and Cd concentrations were not significantly correlated. We suggest that MTLP production may be an important mechanism enabling *Pardosa saltans* populations to persist in ecosystems polluted with heavy metals above a certain level.

Capsule: Spiders from metal-polluted and unpolluted populations show a similar increase in MTLP production when exposed to Cd, with unaffected growth and survival.

Keywords: local adaptation, acclimation, life history, metal, metallothionein

1 INTRODUCTION

Environmental stressors, such as metals, exert strong negative effects on individual fitness in plants, invertebrates and vertebrates, and may ultimately lead to negative population growth rates and a reduced diversity (Klerks and Levinton 1993; Posthuma and Van Straalen, 1993, MacNair, 1997; Shaw, 1999; Burger 2008; Lagisz and Laskowksi, 2008). Metal exposure can decrease individual survival and growth rates either directly, by triggering toxic reactions or eliciting energetically expensive tolerance mechanisms, or indirectly, by reducing prey availability (Calow, 1991; Van Straalen and Hoffmann, 2000; Lock et al., 2001, 2003; Reznick and Ghalambor, 2001). However, some populations are able to persist even under high pollution levels, possibly because divergent selection pressures in polluted sites result in a higher occurrence of genotypes that are locally adapted to metal stress, i.e. have a higher fitness than genotypes originating from non-polluted sites (Klerks and Weis, 1987; Bengtsson et al., 1992; Mouneyrac et al., 2002; Kawecki and Ebert, 2004; Roelofs et al., 2009).

While numerous studies have focused on the effects of metal stress on both animal and plant species, predominantly aquatic, evidence for genetic adaptation to metal pollution in invertebrates remains scarce and is restricted to Oligochaeta, Polychaeta, Gastropoda, Collembola, Isopoda, Ixodida, Aphidoidiea, Lepidoptera and Diptera (Hopkin, 1989; Donker and Bogert, 1991; Bengtsson et al., 1992; Posthuma et al., 1993; Postma et al., 1995; Dallinger, 1996; Aziz et al., 1999; Sterrenborg and Roelofs 2003; Jordaens et al., 2006). Although this scarcity is partly due to practical difficulties (Kawecki and Ebert, 2004), it may also reflect ecological constraints. First, local adaptation to metal stress is believed to impose costs, often linked to energetic trade-offs, which may reduce fitness components of individuals adapted to metal pollution when reared in unpolluted environments, relative to individuals originating from unpolluted populations (Hoffmann and Parsons, 1994; Shirley and Sibly, 1999; Van Straalen and Hoffmann, 2000; Kawecki and Ebert, 2004; Morgan et al., 2007). Second, adaptation through natural selection is generally considered to be slowed down by various opposing forces, especially gene

flow (Reznick and Ghalambor, 2001; but see Bradshaw and McNeilly, 1981; Posthuma and Van Straalen, 1993 and Carroll et al., 2007 for evidence of fast adaptation to metal pollution).

Persistence of a species under environmental stress does, however, not necessarily imply that it is genetically adapted to the stressor. Instead, individuals may have the ability to physiologically acclimate, which is defined as an increased tolerance to the stressor resulting from long-term exposure to sublethal concentrations (Bengtsson et al., 1992; Posthuma and Van Straalen, 1993; Belfiore and Anderson, 1998; Morgan et al., 2007). While genetic adaptation and physiological acclimation are distinct phenomena from a theoretical point of view, under natural conditions these mechanisms are not straightforward to differentiate. Therefore controlled multigenerational studies are required (Klerks and Levinton 1989a; Belfiore and Anderson, 2001; Walker et al., 2001; Lagisz and Laskowski 2008). Both mechanisms have been identified in metal-exposed populations (Klerks and Levinton, 1989b; Bengtsson et al., 1992; Posthuma and Van Straalen, 1993; Shirley and Sibly, 1999; Knapen et al., 2004; Xie and Klerks, 2004). Moreover, plasticity in the expression of tolerance traits itself may be genetically determined, resulting in local adaptation of stress-induced tolerance (Posthuma and Van Straalen, 1993; Via et al., 1995; Roelofs et al., 2006; Morgan et al., 2007; Hendrickx et al., 2008).

A suite of tolerance mechanisms against metal intoxication can be deployed, comprising intake avoidance, decreased influx, increased excretion, and detoxification or sequestration in non-toxic forms (Posthuma and Van Straalen, 1993; Viarengo and Nott, 1993; Bahrndorff et al., 2006; Morgan et al., 2007). Metallothionein-like proteins (MTLPs), in particular, are known to directly bind metals in insoluble fractions (Mason and Jenkins, 1995; Köhler, 2002; Park et al., 2001; Santiago-Rivas et al., 2007). MTLPs are low molecular mass proteins with a strong affinity for metals because of their high cysteine content consisting of sulphydryl-groups that are involved in the transport, detoxification and storage of mainly zinc (Zn), cadmium (Cd) and copper (Cu) (Dallinger, 1996; Nordberg, 1998; Santiago-Rivas et al., 2007; Janssens et al., 2009).

So far, the role of MTLPs in metal tolerance in terrestrial organisms has been studied in collembolans, nematods, isopods, gastropods, earthworms, *Drosophila*, birds and mammals (Dallinger et al., 2000; Znidarsic et al., 2005; Carpene et al., 2007; Hughes and Stürzenbaum, 2007; Vanparys et al., 2008; Janssens et al., 2009). However, less extensively studied invertebrates that play key ecological roles in ecosystems, such as spiders, have also been shown to persist in heavily metal polluted habitats (Salo et al., 1991; Marczyk et al., 1993; Wilczek and Migula, 1996; Wilczek and Babynzska, 2000; Hendrickx et al., 2003). Nevertheless, the importance of the intrinsic functional characteristics of this species group to tolerate high levels of metal exposure remains poorly understood. Spiders are macro-concentrators of metals due to their role as predator and polyphage (Dallinger and Rainbow, 1993; Marczyk et al., 1993; Maelfait and Hendrickx, 1998; Heikens et al., 2001; Hendrickx et al., 2003; Jung et al., 2008). Contrary to insects, which mainly excrete metals (Van Straalen et al., 1987; Janssen et al., 1991; Lindqvist, 1994), detoxification mechanisms in spiders are mainly based on storage in intracellular granules (Brown, 1982; Hopkin, 1989; Janssen et al., 1991; Kramarz, 2000; Wilczek and Babczynska 2000).

We measured MTLP concentrations in response to Cd exposure in *Pardosa saltans*, a ground-dwelling wolf spider (Lycosidae) inhabiting open forests and forest fringes, dominated by *Fagus sylvatica*, *Quercus robur*, *Carpinus betulus* and *Anemone nemorosa* across Europe (Hängi et al., 1995; De Bakker, 2000; Roberts, 1998; Hendrickx et al., 2001). Lycosids are particularly suited to model physiological stress responses as they often reach high densities in severely polluted ecosystems (Wilczek and Babczynska, 2000; Wilczek et al., 2005; Jung et al., 2008). Earlier studies on two other lycosid spiders, *Pardosa amentata* (Kramarz, 2000) and *Pirata piraticus* (Hendrickx et al., 2003), showed linear increases in internal cadmium concentrations during experimental exposure and zero or very low bioelimination rates for Cd, Zn and lead (Pb). Due to their active hunting strategy and predation on Diplopoda, Collembola and Isopoda, that are known to store metals, Lycosids show significantly higher internal metal concentrations compared to other soil-dwelling invertebrates and other spider families inhabiting polluted habitats (Van Hook and Yates, 1975;

Hunter et al., 1987; Larsen et al., 1994; Rabitsch, 1995; Maelfait, 1996; Kohler, 2002; Hendrickx et al, 2004; Wilczek et al., 2004; Jung et al., 2008). To the best of our knowledge, MTLP concentrations in spiders have only been studied in two Lycosids (the closely related *P. lugubris,* and *Xerolycosa nemoralis*; Wilczek, 2005; Wilczek et al., 2008) through flow cytometry based on an immunological reaction.

Since we aimed to study how metal exposure may affect ecologically relevant traits in field populations, we studied effects on individual survival, growth and body mass of spiderlings (see also Donker and Bogert, 1991; Bengtsson et al., 1992; Posthuma and Van Straalen, 1993; Posthuma et al., 1993; Hendrickx et al., 2003; Jung et al., 2005). In spiders, reproductive output is positively correlated with body mass (Hendrickx and Maelfait, 2003; Hendrickx et al., 2003; Hendrickx et al. 2008), which, together with (subadult) survival, determines population growth. By comparing levels of constitutive and Cd-inducible MTLP, and its relation with growth and survival rates, in F1-offspring of *P. saltans* originating from three historically metal polluted sites and three unpolluted reference sites, we here test (i) whether the production of MTLPs is increased under experimental Cd exposure and, if so, whether the response differs between individuals originating from metal polluted and reference sites, and (ii) whether increased MTLP production trades-off with individual growth and survival rates. If historically metal-polluted populations are genetically adapted, we expect higher constitutive MTLP concentrations in individuals originating from these populations compared to individuals from reference populations. If individuals show acclimation, instead, MTLP levels are expected to be inducible upon exposure. However, plasticity in tolerance mechanisms can also be genetically adaptive and in this case individuals from historically polluted populations are expected to show a more inducible or larger increase in MTLP production upon exposure than individuals from unpolluted reference populations.

2 MATERIAL AND METHODS

2.1 Study sites, sampling design and breeding conditions

Study populations of *Pardosa saltans* were located near the villages of Chaudfontaine and La Calamine in the province of Liège (southeast Belgium). The region is known for the presence of natural metal-rich outcrops, however, high local metal concentrations are mainly due to a long history of metal industry, dating back to the 13th century. Zn and Pb extraction peaked during the 19th century and ended in 1970 (Duvigneaud and Jortay, 1987). We selected three polluted and three unpolluted (reference) sites for this study. All three polluted sites were vegetated with plant species adapted to high soil metal concentrations (calamine flora; Bizoux et al., 2004; Graitson et al., 2005; Cappuyns et al., 2006). Within these sites, *P. saltans* preferred patches of isolated *Quercus robus* trees. Prayon (CH-P1) (50°35'N, 5°40'E) and Bois les Dames (CH-P2) (50°35'N, 5°39'E) and are two sites in Chaudfontaine on the east bank of the Vesder river, contaminated due to atmospheric pollution with metal dust, mainly Zn, Pb, Cd and Cu (Graitson et al., 2005). Schmalgraf (CM-P), La Calamine (50°42'N, 6°00'E) is a very small (1.5 ha) site, polluted with Zn, Pb, Cd and Cu through mining activities in nearby sites with natural ore deposits, situated in the Hohnbach alluvial plain, close to the river Gueule (Duvigneaud et al., 1979). For each of these historically polluted sites, a nearby non-polluted (reference) one was selected consisting of recent clearings that were dominated by beech and lacked a metalliferous flora: Louveigné (CH-R1), Chaudfontaine (50°33'N, 5°42'E), Fraipont (CH-R2), Chaudfontaine (50°33'N, 5°44'E), and Kelmis (CM-R), La Calamine (50°42'N, 6°01'E). Another study on metal concentrations in field caught adult *P. saltans* in the same areas showed highest Zn and Cd concentrations in CH-P1 and CH-P2, intermediate in CM-P and significantly lower concentrations in the reference populations (D. Eraly et al., in prep.). Distances between the study populations within the municipalities of Chaudfontaine and La Calamine range between 1 and 4 km, while both municipalities are circa 20 km apart. Since sample areas were small (100 m² maximum) and interspersed with unsuitable habitat for our species (i.e. grasslands, arable fields and urban areas), we consider our populations to

be sufficiently isolated to allow genetic divergence in ecologically relevant traits.

In each polluted and reference site 20 females carrying an egg cocoon were collected by hand picking on 22 and 29 May 2008 and individually transported to avoid mixing of cocoons and mothers. In the lab, each female with attached cocoon was placed in a plastic vial lined with plaster to maintain humidity, and fed with *Drosophila melanogaster* until the offspring hatched or the cocoon was dropped. For populations CH-P1, CH-P2, CM-P, CH-R1, CH-R2 and CM-R, 10, 12, 13, 16, 7 and 16 females produced viable offspring, respectively. Two days after hatching, females and spiderlings were separated and the former were frozen alive (-80°C). For each adult female, a maximum of 20 spiderlings (both males and females) were individually housed in plastic vials (5 cm diameter), lined with a layer of plaster. A total of 1362 offspring (182 of CH-P1, 236 of CH-P2, 211 of CM-P, 306 of CH-R1, 132 of CH-R2 and 304 of CM-R) was reared in incubators at 20°C on a 16h:8h light-dark regime and fed with collembolans (*Sinella curviseta*) ad libitum.

2.2 Exposure experiment

From the third instar stage onwards, half of the offspring of each female from the six populations received the Cd exposure treatment. They were fed with flight-deficient fruit flies, *Drosophila melanogaster* that had been raised on a dogfood-oat-banana medium that was contaminated with 100 µg/g ww Cd, added as a $Cd(NO_3)_2$ solution, for a minimum of three days (+Cd treatment) (Mayntz and Toft, 2001; Hendrickx et al., 2003). The other half of the offspring were fed with flies raised on a similar, Cd-free medium (-Cd treatment). Twice a week, experimental and control individuals were fed two, two, three or four flies depending whether they were third, fourth, fifth or sixth instar, respectively, and moulting and survival data were recorded. At an age of 35 and 50 days after hatching, all individuals were weighed to the nearest 0.1mg (Galaxy™ 110 Ohaus) to determine mass increase (N = 851). Growth rate was also expressed as the number of days before reaching the sixth instar. Three to four days after reaching this

stage, all spiderlings were sexed, frozen in liquid nitrogen and stored at -80 °C. It took between 59 and 106 days, with an average of 84, to reach the sixth instar stage, which was also the time of exposure.

2.3 Metal analysis and Metallothionein-like protein (MTLP) measurement

Cd, Zn, Cu and MTLP concentrations were measured in a total of 96 offspring from populations CH-P1 (n=16), CH-P2 (n=18), CM-P (n=16), CH-R1 (n=16), CH-R2 (n=15) and CM-R (n=15). One experimental and one control offspring with comparable weights were selected from each female. Because MTLP-concentrations and relationships with metal body burden were earlier shown to be sex-specific (Wilczek et al., 2008), we restricted our analyses to female offspring. Although spiders were experimentally exposed to cadmium only, we also measured Zn and Cu concentrations as a control for our treatment effect and because these metals are also bound by MTLP. After recording the fresh weight of each spiderling (Mettler Toledo, AT261, Deltarange), we added 350 µl of buffer A (10 mM tris HCl and 85 mM NaCL, pH 7.4, Sigma-Aldrich, USA) and homogenized each individual during one minute on ice in new polypropylene vials with a Tissue Ruptor (Qiagen). After extracting 100 µl for MTLP measurement, samples were retained for measuring the metal concentration.

To measure internal metal concentrations, the homogenate was dried at 60°C for 48 hours and a nitric acid (70%, Merck, Pro Analysis, Germany)/hydrogen peroxide (30%, Merck, Pro Analysis) microwave digestion (Blust et al., 1988) was performed. All samples were diluted with Milli-Q water (Millipore, USA) and weighed again. Cd-, Zn- and Cu-concentrations were measured using ICP-MS (Varian Ultra Mass 700, Australia). Certified mussel reference material (CRM 278) of the EU Community Bureau of Reference was used as a quality control. All recoveries were within 10% of the certified values. Metal concentrations were expressed as µg/g wet weight.

To measure MTLP concentrations, the 100 µl samples were centrifuged (20min, 13200g, 4°C; Eppendorf Centrifuge 5804R, Germany), 50 µl of the

supernatans was separated and stored at -80 °C until analyzed. The highly MTLP-specific cadmium saturation thiomolybdate assay (Klein et al., 1994) was applied. During this process, oxidized MTLP is converted to native MTLP with the reducing agent 2-mercaptoethanol and Zn^{2+} as metal donor, followed by saturation with the radioactive Cd^{109} isotope. Cd^{109} concentrations were then quantified with a Minaxi-Autogamma 5530 counter (Canberra Packard, USA). For the Cd-MTLP concentration calculation, a total saturation of MTLP with the metal ions at a ratio of 7 mol Cd per mol MTLP, as has been demonstrated for vertebrates and most invertebrates, was assumed (Kito et al., 1982; Viarengo and Nott, 1993; Hensbergen et al., 2000; Dabrio et al., 2002). MTLP concentrations were expressed as nmol/g wet weight.

2.4 Statistical analysis

Because we aimed to compare MTLP-concentrations and life-history traits between historically metal-polluted and reference populations, naturally polluted (CH-P1, CH-P2 and CM-P) and reference populations (CH-R1, CH-R2 and CM-R) were nested within a 'polluted' and 'reference' group respectively, and the factor 'pollution' was modeled as fixed effect in all analyses. To account for variation within each pollution group, however, population and its interaction with Cd-treatment were modeled as random effects nested within each group. We used general linear mixed models (proc mixed; SAS 9.1, SAS Institute Inc. ©) to test for differences in Cd-, Zn-, Cu- and MTLP-concentrations between polluted-reference groups and Cd-treatments, and to model pollution*treatment interactions. Prior to statistical analysis, Cd -concentrations were ln-transformed to fulfill the assumptions of homoscedasticity (Levene's test) and of normal distribution of the residuals (Shapiro Wilk test). We used general linear mixed models to test for relationships between MTLP and Cd concentrations within the +Cd and -Cd treatment group separately and to compare these relationships between historically-polluted and reference groups. To determine the estimated amount of Cd in the spider that is not bound to MTLP (free Cd, mol), we subtracted the internal molar Cd concentration from the maximum

Cd-binding capacity (as derived from the thiomolybdate assay). These were compared between treatments and both pollution groups.

We used general linear mixed models to test effects of cadmium exposure (treatment), population history (polluted versus reference groups), and the two-factor interaction on individual growth rates (mass increase and time to reach the sixth instar). Since about 20 offspring per mother were tested, the identity of the latter (nested within population) was modeled as a random effect As both males and females were included in the analysis, factor 'sex' and its interaction with the main effects were modeled as fixed effects.

Finally, we used generalized linear mixed models (proc glimmix; SAS 9.1, SAS Institute Inc. ©) with logit link function to test for differences in the proportion of offspring surviving till the sixth instar stage between groups.

3 RESULTS

3.1 Cadmium and other metal concentrations

Individuals under +Cd treatment faced a strong increase in average total Cd body burden compared to individuals from the control treatment (Table 1; $F_{1,3.73} = 1928.91$; $p < 0.0001$). However, the effect of the Cd treatment did not differ between polluted and reference populations (Table 1; $F_{1,3.73} = 0.00$; $p = 0.95$), nor did these groups differ in average Cd concentration (Table 1; $F_{1,4.27} = 0.59$; $p = 0.48$). There were no significant differences between all population and exposure combinations in average Cd concentration (estimated variance in mean Cd concentration respectively 0 and 0.011 ± 0.018).

Treatment	-Cd		+Cd	
Population	Cd conc (µg/g ww)	N	Cd conc (µg/g ww)	N
CH-P1	0.30 ± 0.10	8	55.60 ± 15.47	7
CH-P2	0.48 ± 0.48	9	62.82 ± 6.95	9
CM-P	0.39 ± 0.44	8	53.46 ± 13.07	8
CM-R	0.74 ± 0.92	7	52.76 ± 8.83	8
CH-R1	0.36 ± 0.14	8	64.68 ± 16.78	8
CH-R2	0.27 ± 0.07	8	67.63 ± 16.81	8
Average-P	0.39 ± 0.37	25	61.10 ± 13.30	24
Average-R	0.45 ± 0.53	23	57.68 ± 14.46	24

Table 1 | Cd concentrations (mean ± standard deviation) measured in experimentally exposed (+Cd) and control (-Cd) offspring originating from different populations. "P" refers to populations from polluted sites, "R" to populations from reference sites. See text for details.

Although spiders were not experimentally exposed to Cu and Zn, the concentrations of these metals were significantly higher in the control treatment, regardless of whether individuals originated from polluted or reference populations (Table 2). Populations within the reference and polluted group and the treatment*population group differed, greatly in average Zn concentration and less in average Cu concentration (Table 2).

						mean conc ± SE	
	Factor	numDF	denDF	F	P	-Cd treat	+Cd treat
Zn	treatment	1	4.29	138.93	0.0002	59.57±14.74	46.42± 8.85
	pollution	1	3.95	0.01	0.92	/	/
	treat*pollution	1	4.29	0.11	0.76	/	/
	Variance ± SE	population: 15.07±17.01; population*treat: 0					
Cu	treatment	1	4.05	17.65	0.01	20.59±4.67	17.49±3.70
	pollution	1	3.97	0.18	0.70	/	/
	treat*pollution	1	4.05	1.03	0.37	/	/
	Variance ± SE	population: 0.55 ± 1.18; population*treat: 0					

Table 2 | Differences in Cu and Zn concentrations between polluted and reference populations ("pollution") and experimental Cd-treatments ("treat"; + or -Cd) as obtained from general linear mixed models. Variance estimates refer to the estimated variance in means of random factors; means (±SE) are listed for significant effects only.

3.2 MTLP concentrations

When exposed to Cd, individuals contained on average 12.30 ± 0.41 nmol MTLP/g fresh weight, compared to 7.75 ± 0.41 nmol MTLP/g fresh weight for individuals of the control treatment ($F_{1,3.53} = 97.34$; $p = 0.0011$; Fig. 1). The increase in MTLP concentrations when exposed to Cd did not differ between individuals from polluted and reference populations (Fig 1; $F_{1,3.53} = 0.11$; $p = 0.76$), nor did the average MTLP concentration differ between these groups (Fig 1; $F_{1,4.36} = 0.23$; $p = 0.66$). Covariance within the populations and within treatment*population group was not siginificantly different from zero (variance ± SE: 0.44 ± 0.57 and 0, respectively).

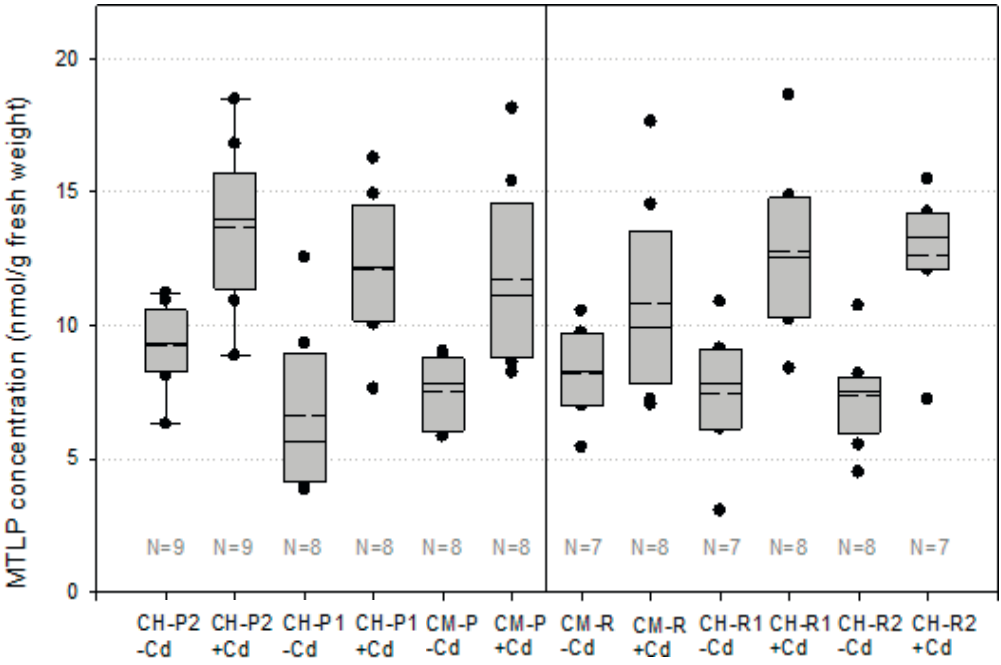

Figure 1 | Boxplot diagram of MTLP concentrations in spiders from polluted (-P) and reference (-R) populations and subjected to Cd (+Cd) and control (-Cd) treatments (see text for details). (line= average; box= upper and lower quartiles, whiskers: 90th and 10th percentiles; dots = extreme values).

Under Cd exposure, there was no significant correlation between MTLP and Cd concentration ($F_{1,35.8}$ = 0.79; p = 0.38), nor did the direction or the strength of the relationship differ between individuals originating from polluted and reference populations ($F_{1,35.8}$ = 0.01; p = 0.94). MTLP and Cd concentrations were not correlated within the control group ($F_{1,43.4}$ = 0.5; p = 0.48), nor did the direction or the strength of the relationship differ between individuals originating from polluted and reference populations ($F_{1,41.9}$= 0.02; p = 0.89).

There was a significant difference in the estimated amount of free Cd between treatments ($F_{1,8.78}$ = 563.95; p < 0.0001). In the exposed individuals there was no estimated free Cd (-0.44 ± 0.014 µmol), while this

amount was 0.050 ± 0.013 µmol in the unexposed treatment. No differences between pollution groups ($F_{1,8.83} = 0.99$; $p = 0.35$) or effects of treatment between populations ($F_{1,8.08} = 0.74$; $p = 0.41$) were present. The level of variation in mean estimated free Cd among populations within each group of origin was estimated as zero, while the level of variation within the treatment*population groups was negligible (0.00039 ± 0.00063.)

3.3 Growth rate

Our data did not reveal differences in mass increase (day 35 to 50) between Cd-treatments ($F_{16.34} = 2.16$; $p = 0.19$) nor pollution groups ($F_{1,64.3} = 0.70$; $p = 0.41$). Likewise, effects of Cd-treatment on growth did not differ between polluted and reference populations ($F_{1,5.13} = 5.13$; $p = 0.97$). However, females gained significantly more weight, irrespective of treatment or pollution history (females: 3.31± 0.13 mg, males 2.41 ± 0.13 mg; $F_{1,799}=121.44$; $p < 0.0001$). Variation among mothers was relatively high (0.92 ± 0.18), indicating that offspring originating from different mothers differed substantially in growth. Due to this large variation, variation among populations and population*treatment was not detectable larger than zero (0 and 0.0025 ± 0.011, respectively).

Time to develop into the sixth instar did not differ between treatments ($F_{1,535} = 0.26$; $p = 0.61$) nor between both pollution groups ($F_{1,66.2} = 0.17$; $p = 0.68$). However, females reached the sixth instar earlier (76.00 ± 0.63 days) than males (77.87 ± 0.71 days) ($F_{1,554} = 12.15$; $p = 0.0005$).

3.4 Survival

Neither treatment ($F_{1,8} = 1.87$; $p = 0.21$), nor pollution group ($F_{1,4.1} = 0.64$; $p = 0.47$) or their interaction ($F_{1,8} = 1.01$; $p = 0.35$) could be shown to affect the cumulative survival of spiders between the third and sixth instar (Fig 2). Variation among populations was low (0.13 ± 0.11).

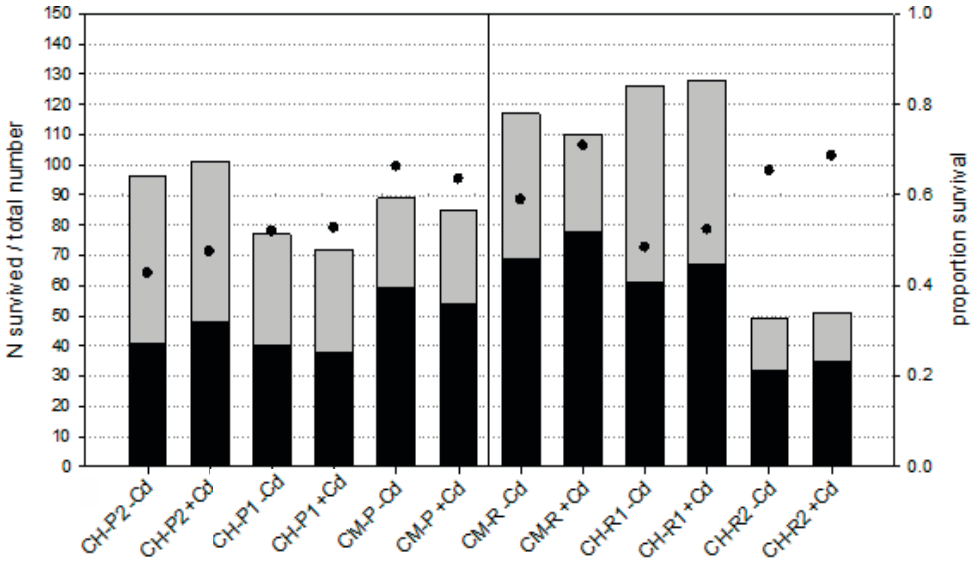

Figure 2 | Survival from 3rd to 6th instar in individuals of polluted (-P) and reference (-R) populations, both exposed to a Cd and control treatment. Black bars represent the number of spiders that survived; grey bars the number that died. Average proportion survival is represented by dots.

4 DISCUSSION

Experimental results from this study point towards a plastic response in MTLP production to metal exposure in *Pardosa saltans*. For none of the response variables under study, individuals from the studied historically-polluted populations showed a stronger response to the cadmium exposure than naive individuals from unpolluted populations. Although cadmium concentrations in the treatment group were strongly elevated compared to the control group, there was no measurable effect on survival or growth. It is unlikely that the absence of effects is due to cadmium body burdens that are too low since Cd concentrations in exposed spiders were on average three times higher than those measured in field caught *P. saltans* from the most polluted site (D. Eraly et al., in prep.) and than in other studies on wolf spiders (Hunter 1987; Rabbitsch 1995; Heikens 2001; Wilczek 2000, 2004) or arachnids (Wilczek 1996; Hendrick et al. 2003; Jung 2007), taken into account a fresh to dry weight ratio of about 4 in this species. In the

P. saltans field populations clear effects on certain life history traits, like adult body size and fecundity under polluted conditions were present (D. Eraly et al., in prep.). Heavy metal ecotoxicological data of metals on spiders are very scarce (Hendrickx et al. 2003; Wilczek et al. 2004; Jung 2005; Wilczek 2005). In *Pardosa astrigera*, lethal body concentrations were estimated at 1050 mg Cd/kg DW (Jung et al. 2007), which is about four times higher than the highest concentrations measured in this study.

Absence of measurable effects of cadmium treatment on *P. saltans* survival suggests the existence of one or more effective tolerance mechanisms against cadmium intoxication. First, metallothionein-like protein production increased 1.6 fold when individuals were experimentally exposed to cadmium. These proteins are known to effectively bind metals and make them biologically unavailable, and may therefore protect individuals against adverse effects of metal intoxication (Mason and Jenkins, 1995; Dallinger, 1996; Nordberg, 1998; Santiago-Rivas et al., 2007; Park et al., 2001). Moreover, this increase enabled the spiders to bind more Cd, since the estimated amount of free Cd was lower in exposed spiders than in the control treatment, suggesting the presence of a particular cadmium threshold concentration for increased MTLP production. Since other metals are also bound by MTLP and the metal stoichiometry of MTLP in our species remains unknown, results from this study only provide relative measures for comparison and likely underestimate the absolute values. Second, levels of metal intake seemed to decrease under increasing exposure, as was apparent from the lower Cu- and Zn-concentrations under Cd treatment. Unlike Cd, for which concentrations were too high to allow a reduction in intake to normal levels, individuals were not experimentally treated with Cu and Zn. However, the reduction in concentration was relatively low and unlikely to be biologically significant. Third, while Cd concentrations in individuals from the treatment group were very high, some of the Cd may have been (partly) redistributed to less vital tissues (Hensbergen et al. 2000; Wilczek and Babczynska, 2000; Desouky, 2006; Morgan, 2007). Moreover, an interaction between the different detoxification mechanisms is very plausible, with metals initially bound to MTLP subsequently being redistributed into insoluble metal rich granules through the lysosomes (Posthuma and Van Straalen, 1993; Viarengo and

Nott, 1993; Mason and Jenkins, 1995; Desouky, 2006; Morgan et al., 2007). Because entire individuals were sacrificed when sampling body burdens, and metal rich granule concentrations were not measured in this study, the latter processes remained unconfirmed. However, given the large individual variability and the relatively low number of analyzed females per pollution group*exposure combination, increased sample sizes could eventually reveal more subtle patterns in population specific responses.

The absence of a measurable reduction in growth or survival rates upon exposure in all of the study populations questions the (energetic) cost of tolerance as has been suggested in other studies (Morgan et al., 2007). As we only quantified the actual difference in MTLP concentration and not the turnover rate, the actual MTLP production could even be underestimated (Mouneyrac et al., 2002; Amiard et al., 2006). Contrary to what is generally assumed, various tolerance mechanisms comprise modifications of energetically cheap metal-regulation mechanisms, while apparent energetic trade-offs between tolerance for metals and other life-history traits may also result from antagonistic pleiotropy (Calow, 1991; Posthuma and Van Straalen, 1993; Van Straalen and Hoffmann, 2000; Morgan et al., 2007). Along the same lines, some species of Isopods and Collembolans showed more, rather than less, vigorous life history traits in populations exposed to metal pollution, probably as a result of direct selection on life-history characteristics (Posthuma and Van Straalen, 1993).

This species seems to be able to show an efficient and plastic tolerance mechanism towards cadmium exposure even when it has not been historically exposed to large concentrations of this metal. Phenotypic plasticity allows individuals to cope with a wider range of environmental factors and with variability or unpredictability, a characteristic that is most advantageous in a heterogenic environment with migration between patches (Sultan and Spencer, 2002; Terblanche and Kleynhans, 2009). Although only a limited number of studies have examined the costs of plasticity in animals (Hoffmann, 1995; Dewitt et al., 1998; Hoffmann and Hewa-Kapuge, 2000; Steiner and Buskirk, 2007; Kristensen et al., 2008; Terblanche and Kleynhans, 2009; Van Buskirk and Steiner, 2009), they are usually found to be relatively low on a global scale. When migration exists,

costs can be moderate on a local scale and still allow the plastic genotype to have a higher fitness in the metapopulation and persist instead of the locally specialized genotype (Sultan and Spencer, 2002).

We could not find any indication for local genetic adaptation to metal exposure in this species, as has been noticed for other studies (reviewed in Belfiore and Anderson, 2001). While absence of local genetic adaptation may be due to gene flow, we doubt whether *P. saltans* was able to disperse between the different study populations given its inability to perform aerial dispersal under experimental conditions (D. Eraly, unpubl. data). Moreover, if gene flow would be present, strong local selection caused by metal pollution (Klerks and Weis, 1987; Posthuma and Van Straalen, 1993; Shaw, 1999; Shirley and Sibly, 1999; Morgan et al., 2007) may still allow populations to differentiate in genetically determined ecologically relevant traits (Schluter, 2001; De Wolf et al., 2004; Hey, 2006; Dhuyvetter et al., 2007; Morgan, 2007; Nosil, 2008).

As we confined our study to MTLP production, local adaptation in other tolerance mechanisms (like biomineralisation, reduced uptake, increased efflux, detoxifying enzymes) still remains possible, especially since some have been demonstrated in the very closely related lycosid *P. lugubris*, though adaptation in these mechanisms was not explored (Wilczek et al., 2004). However, even if constitutive expression of tolerance mechanisms in metal exposed populations would be present, they apparently do not exert strong negative effects on growth and survival.

Conclusions

Although cadmium exposure elevated internal burden and MTLP concentration substantially in *Pardosa saltans*, no negative effect was observed on juvenile growth and survival. Contrary to the expectations, spiders originating from metal polluted and reference sites responded in a similar way. Our results suggest that the ability of a plastic response in MTLP production serves as an efficient mechanism to reduce effects of metal exposure on individual growth and survival and can be one of the mechanisms allowing this species to occur in high densities in ecosystems that are radically altered by metal contamination.

Author's contribution

Debbie Eraly and Frederik Hendrickx conceived and designed the study and wrote the manuscript together with Luc Lens. Debbie Eraly prepared the samples for analysis under supervision of Lieven Bervoets. Debbie Eraly and Frederik Hendrickx analyzed the data.

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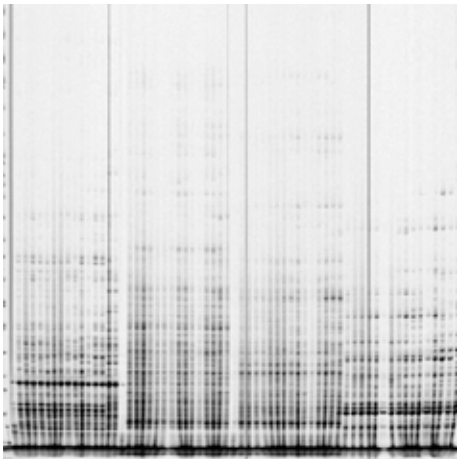

It makes things more interesting when you read between the lines

CHAPTER 3 | ASSESSING GENETIC DIVERGENCE IN A METAL-EXPOSED WOLF SPIDER POPULATION USING AFLP GENOME SCANS

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ABSTRACT

Organisms are exposed to various pollutants of which metals comprise one of the most persistent ones. If the degree of metal exposure has strong detrimental effects on fitness, selection for more tolerant genotypes is expected. However, it remains less understood how exposure and adaptation to metal stress may affect population genetic variation at a genome wide scale. First, if selection is very strong, this may lead to population bottlenecks and founder events and eventually lead to a pattern of genome-wide genetic erosion. Second, if selection acts on a few loci only and gene flow is not impeded between exposed and unexposed populations, populations are expected to be differentiated at a few loci only (i.e. outlier loci). Third, if gene flow between exposed and unexposed populations is restricted, e.g. due to selection against migrants, differentiation between ecotypes is expected at a genome wide scale and overall genetic variation at the metapopulation level is not expected to decrease. We investigated these hypotheses on 6 field populations of the wolf spider *Pardosa saltans* originating from both metal polluted and reference sites using AFLP markers. We found larger differentiation between polluted and reference populations than between populations within these groups, but no evidence for genetic erosion due to metal exposure. Using two methods (Bayescan and SAM), we discovered several outlier loci that are linked to metal exposure and need further investigation to define the genes involved. The results from these analyses not only revealed signs of genetic differentiation indicative of local adaptation, bur moreover suggest that metal pollution in this metapopulation selects identical alleles in the face of homogenizing gene flow.

Keywords: local adaptation, genetic erosion, AFLP, outlier loci, Bayescan, Spatial Analysis Method

1 INTRODUCTION

Organisms are exposed to various pollutants of which metals comprise one of the most persistent ones, with strong direct and indirect impacts on individual fitness in both plants and animals (Hunter *et al.* 1987a; Hunter *et al.* 1987b; Klerks & Levinton 1993; Macnair & Christie 1983; Posthuma & Van Straalen 1993; Das *et al.* 1997; Bertin & Averbeck 2006; Burger 2008; Lagisz & Laskowski 2008; Morgan *et al.* 2007). However, some populations are able to persist even under high pollution levels. One of the underlying mechanisms is ecological adaptation, where divergent selection pressures in polluted sites result in a higher occurrence of genotypes that are locally adapted to metal stress. Ultimately, ecological adaptation may lead to genetic differentiation in traits between exposed and non-exposed sites (Klerks & Weis 1987; Bengtsson *et al.* 1992; Hendrickx *et al.* 2001; Mouneyrac *et al.* 2002; Kawecki & Ebert 2004; Roelofs *et al.* 2009).

For ecological adaptation to take place, standing levels of genetic variation should be sufficiently high (Frankham *et al.* 2010) and levels of gene exchange with non-exposed populations sufficiently low (Slatkin 1987; Barrett & Schluter 2008; Schluter & Conte 2009). Due to the fact that gene flow is rarely absent (i.e. low numbers of migrants are thought to be sufficient to retain genetic connectivity and oppose population differentiation; Lowe et al. 2005), adaptation through natural selection is generally considered to be a relative slow process (Reznick & Ghalambor 2001). In particular, the co-existence of adapted and non-adapted populations would either require a strong restriction on gene flow (Rasanen & Hendry 2008) or the evolution of ecological by-product mechanisms. However, when selective pressures are sufficiently strong, adaption may also occur in the presence of relatively high gene flow, causing "isolation by adaptation" rather than "isolation by distance" (Dhuyvetter *et al.* 2007; Nosil *et al.* 2007; Nosil *et al.* 2009). Still, the few studies that addressed this topic produced equivocal results (Winterer & Weis 2004; Castellano *et al.* 2012).

Evidence for genetic adaptation to metal pollution in invertebrates is growing (Klerks & Weis 1987; Postma *et al.* 1995; Dallinger 1996; Martinez & Levinton 1996; Mouneyrac *et al.* 2002; Sterenborg & Roelofs 2003;

Morgan *et al.* 2007; Hendrickx *et al.* 2008 ; Roelofs *et al.* 2009 Costa *et al.* 2012) Though, the effect of metal stress on genetic differentiation and diversity on a genome wide scale remains poorly explored altogether (Van Straalen & Timmermans 2002). Yet, recent theoretical and molecular advances in population genetics currently allow to search and even identify loci subjected to divergent selection, which provides a unique opportunity for studying natural selection in action (Van Straalen & Timmermans 2002; Joost *et al.* 2007; Foll & Gaggiotti 2008; Williams & Oleksiak 2008; Hohenlohe *et al.* 2010).

Recently, different, mutually non-exclusive hypotheses have been put forward regarding putative genetic consequences when populations are subjected to different levels of stress.

First, if sufficient genetic variation is present at particular loci to increase metal resistance, strong genetic divergence at these loci can be expected. This may lead to relatively stronger differentiation at these (and closely linked) loci compared to the rest of the genome, leading to genomic islands of divergence (Storz 2005; Nosil *et al.* 2007; Paris *et al.* 2010). These so called outlier loci are potential signatures for adaptation and may provide help to identify key genes in (micro-)evolutionary process of speciation (Bonin *et al.* 2006; Joost *et al.* 2008; Hohenlohe *et al.* 2010). Scanning the patterns of DNA polymorphism at the genomic level enables to evaluate the amount of neutral genetic diversity and to identify these outlier loci since they behave differently from the rest of the genome. Selection may be the underlying cause of their atypical behavior either because they are direct targets of selection or because they are genetically linked to a selected locus (Storz 2005; Nosil *et al.* 2007; Paris *et al.* 2010). Detection of loci subjected to divergent selection by comparing the degree of differentiation across the genome has the advantage that no a priori knowledge about the function of the genes is required (Storz 2005; Nosil *et al.* 2007). Computer simulations can be used to model the behavior of neutral loci under a defined evolutionary scenario, and loci lying outside the neutral distribution are detected as outliers (Beaumont & Balding 2004; Bonin *et al.* 2006; Egan *et al.* 2008). Therefore a large number of genetic markers distributed throughout the entire genome should be scored

in individuals from different environments. A powerful application of the genome scan approach uses replicated comparisons of different types of population pairs. The advantages of this approach especially compared to QTLmapping are (i) increasing ease to generate large numbers of genetic markers, (ii) no strict need to obtain information on quantitative traits; (iii) ease of sampling individuals, without having to know their breeding history (Stinchcombe & Hoekstra 2008).

Second, if long periods of ongoing divergent selection result in fitness reduction of hybrids due to recombination and maladaptation, it can be expected that this results in selection for reproductive isolation (i.e. reinforcement or selection against hybrids). As such, gene flow among ecologically divergent populations may become restricted while exchange of gene flow may persist between populations subject to comparable selection pressure. Under such scenario, differentiation between ecotypes (groups of populations, distinguished by a composite of variation in different traits and allele frequencies across loci over space, Lowry 2012) can be expected to occur also for neutral genes scattered throughout the genome and hence not to be restricted to those that are directly involved in adaptation (Wu 2001). If so, overall genetic variation at the metapopulation level would not be expected to decrease (Van Straalen & Timmermans 2002).

Third, if selection against maladapted individuals is very strong, only a few individuals might be able to survive in stressful environments, subsequently leading to population bottlenecks and founder events (Van Straalen & Timmermans 2002). All these mechanisms can eventually lead to a pattern of genome-wide genetic erosion, hence an overall decrease in genetic diversity of exposed populations compared to unexposed (i.e. reference) populations (Van Straalen & Timmermans 2002; Dibattista 2008). To study genetic erosion it is especially important to use genome wide markers. Since standing levels of genetic variation affect the potential for future adaptations, a decrease therein may limit long-term survival prospects. The bottleneck effect is responsible for a pattern of genetic erosion when very strong selection only allows tolerant individuals to survive. Due to founder effects, with only a limited number of

individuals colonizing polluted sites, these can also have a low genetic diversity. Bottlenecks may effectively lead to genetic erosion if very strong selection only allows tolerant individuals to survive. Pollution may decrease genetic variation also through random genetic drift and inbreeding, particularly when population size decreases due to increased mortality (Posthuma & Van Straalen 1993; Belfiore & Anderson 2001; Schellhorn *et al.* 2004; Keane et al. 2005). In a recent review of Dibattista (2008) however, pollution in general in some cases could also increase genetic variation rather than decrease it. The increase can be explained by an increased mutation rate (Baker *et al.* 2001) or selection for heterozygotes (overdominance hypothesis, Bickham *et al.* 2000). The absence of a reduction can be explained by the fact that the conditions for genetic erosion i.e. no gene flow and a very strong selection pressure, are not always likely to occur.

In this study, we observe genome-wide signatures of metal adaptation by quantifying the degree of differentiation for a large number of AFLP loci among three metal-exposed and three metal-unexposed (reference) populations of the wolf spider *Pardosa saltans*. The following research questions are addressed: (i) Is genetic diversity lower in polluted populations; (ii) Is genetic differentiation larger between polluted and reference populations compared to between populations of the same state of pollution; (iii) Did some loci undergo selection through metal stress as inferred from the relative presence of outlier loci in polluted (compared to reference) populations; (iv) Is there a relationship between the frequency of outlier loci and the level of metal pollution.

2 MATERIAL AND METHODS

2.1 Study system

The study species, *Pardosa saltans*, is a ground-dwelling wolf spider (Lycosidae) inhabiting open forests and forest fringes, dominated by *Fagus sylvatica, Quercus robur, Carpinus betulus* and *Anemone nemorosa* across Europe. It occurs at large densities in forest clearings (Hänggi et al., 1995;

Roberts, 1998; De Bakker et al., 2000; Hendrickx et al., 2001). Lycosids are particularly suited to model physiological stress responses as they often reach high densities in severely polluted ecosystems (Wilczek & Babczynska 2000; Wilczek *et al.* 2005; Jung *et al.* 2008). Lycosids show significantly higher internal metal concentrations compared to other soil-dwelling invertebrates and other spider families inhabiting polluted habitats (Vanhook & Yates 1975; Hunter *et al.* 1987b; Larsen *et al.* 1994; Rabitsch 1995; Maelfait 1996; Kohler 2002; Hendrickx *et al.* 2004; Wilczek *et al.* 2004; Jung *et al.* 2008; Eraly *et al.* 2011).

Study populations of *Pardosa saltans* were located in the province of Liege (southeast Belgium). The region is known for the presence of natural metal-rich outcrops of zinc and lead that were industrially extracted since the 13th century. Extraction peaked during the 19th century and ended in 1970 (Duvigneaud & Jortay 1987). Polluted sites were initially located based on a study on the distribution of *Viola calaminaria* (Gingins), a typical metalliferous plant species that grows on soils with average soil metal concentrations of 11.886 mg/kg Zn, 24.3 mg/kg Cd and 9.342 mg/kg Pb (Bizoux *et al.* 2004). Metal body burdens of *P. saltans* on this site were determined in an earlier study and found to differ profoundly between polluted and reference sites (Eraly *et al.* 2011 and *Table 2*). Three contaminated areas with high densities of *P. saltans* were selected as study sites. Bois les Dames (CH-P2, 50°35'N, 5°39'E) and La Rochette à Prayon Pond (CH-P1, 50°35'N, 5°40'E), both in Chaudfontaine, are located at the east bank of the Vesder river and contaminated due to atmospheric pollution with metal dust (Graitson & Goffart 2005). Schmalgraf, La Calamine (CM-P, 50°42'N 6°00'E) is a very small (1.5 ha) site in the Hohnbach alluvial plain, close to the river Gueule, polluted through mining activities in nearby sites with natural ore deposits (Duvigneaud J. *et al.* 1979). All three sites are sparsely vegetated with a metal-adapted calamine flora (Bizoux *et al.* 2004; Graitson & Goffart 2005). Within these sites, *P. saltans* preferred patches of isolated *Quercus robur* trees. For each of these historically polluted sites, a nearby non-polluted (reference) site was selected consisting of recent clearings that were dominated by beech and lacked a metalliferous flora: Louveigné (CH-R1, 50°33' N, 5°42' E) and Fraipont (CH-R2, 50°33' N, 5°44' E), both in Chaudfontaine, and Kelmis, La

Calamine (CM-R, 50°42′ N,6°10′ E). Distances between the study populations within the municipalities of Chaudfontaine and La Calamine ranged between 1 and 4 km, while both municipalities were circa 20 km apart. The study populations occur in a mosaic of both historically contaminated sites exposed to a mixture of metals as Cd, Zn, Pb and Cu and reference populations (Eraly *et al.* 2011). Earlier studies on field populations of this species showed negative relationships between body mass, condition, reproductive output and allocation and Cd body burden. However, no differences between polluted and reference populations were observed for the production of metallothionein like proteins (MTLP), often considered as an important metal defense mechanism (Eraly *et al.* 2011). Female spiders were collected alive by hand picking in May 2007 and April 2008 in the six populations.

2.2 AFLP analysis

To address our research questions we used the technique of Amplified Fragment Length Polymorphism. During the last decades AFLPs have been widely applied to study the genetic structure of natural populations, yet are still somewhat underused in studies of animal populations (Bensch & Akesson 2005). The technique entails digestion of DNA by specific sets of enzymes and ligation of a template for PCR primers (Vos *et al.* 1995). This technique produces a banding pattern depending on the length of the different fragments and allows to screen for a large amount of a markers, distributed throughout the genome, relatively easy and inexpensive without prior knowledge on the genome studied (Bensch & Akesson 2005; Meudt & Clarke 2007). It is a dominant marker and theoretically AFLP is expected to screen for both neutral and quantitative or selective loci. This is necessary for detecting outlier loci linked to selection in genome scans. AFLP analysis has been proven useful to study genetic diversity in spiders although only a limited number of studies have been published (Bilde *et al.* 2005; Smith *et al.* 2009; Lambeets *et al.* 2010).

After collection, spiders were kept a few days in the laboratory and were frozen alive in liquid nitrogen and homogenized to fine powder with a mill (Retsch MM 200) at 30 Hz during 3 min just prior to DNA extraction. DNA was extracted from the thorax and legs using the DNeasy Blood and Tissue

Kit (Qiagen) according to the manufacturer's protocol. DNA quality and concentration were controlled on 1.5% agarose gels. For further AFLP analysis 100 ng of DNA was used according to Vos *et al.* (1995). Restriction and ligation was performed in a single step. Amplification of fragments was performed in two steps using the primer combinations PstI + A/MseI + A for preamplification and four combinations (PstI + AGT/MseI + ACC, PstI + ACT/MseI + ACC, PstI + ACT/MseI + AGA, PstI + ACT/MseI + AGG) for selective amplification. Fragment separation and detection took place on a Nen IR2 DNA analyzer (Licor) using 36 cm denaturing gels with 6.5% polyacrylamide. IRDye size standards (50–700 bp) were included for sizing of the fragments. Only clear, intense bands that were easily discernible were scored. Scoring was done using the SAGAmx software (Licor) and only polymorphic bands were scored, allowing to quantify relative differences between different populations.

2.3 Statistical Analyses

Genetic diversity within populations was estimated as the number and percentage of polymorphic loci per population and expected average heterozygosity (Hj) and genetic differentiation between populations as Nei's D and F_{st} using the program AFLP-SURV under the assumption of Hardy-Weinberg equilibrium (Weir & Cockerham 1984; Lynch and Milligan 1994; Zhivotovsky 1999; Vekemans *et al.* 2002). For AFLP, being dominant markers, HWE can not be determined, but earlier allozyme studies in some of the study populations found them to be in HW-equilibrium, though this meausere can be marker-dependent. Between-population differences were based on allele frequencies at each locus and inferred from a Bayesian method with non-uniform prior distribution of allele frequencies (Zhivotovsky 1999). As an alternative measure to pairwise F_{st}, Phi_{PT} was estimated using Analysis of Molecular Variance (AMOVA) with the Microsoft Excel add-in program GenAlEx6 (Peakall & Smouse 2006), using 999 permutations and interpolating missing data. Phi_{PT} measures the similarity of pairs of individuals drawn at random from the same populations, relative to pairs of individuals drawn from the total sample (analogous to F_{st}). significance values are also calculated and Bonferroni corrected

(considered significant when $p<0.0033$ (0.05/numer of pairwise comparisons, 11 in this case)).

To test whether metal exposure resulted in a higher degree of genetic differentiation among differentially exposed populations, we compared the degree of genetic differentiation among clusters of populations to those within clusters based on an AMOVA modeling procedure as implemented in GenAlEx6 (Peakall & Smouse 2006). Analyses were performed by clustering populations either by geographic cluster (CH vs KM populations) or pollution level (-P vs -R populations). Using this method allowed to hierarchically partition variation at different levels based on Euclidean pairwise genetic distances. We used the index Phi_{RT} to express the level of similarity between pairs of individuals drawn at random from the same cluster compared to individuals drawn at random from the total sample, and hence, to quantify the percentage of variance partioned among clusters. Likewise, PHI_{PR} reflected the correlation among individuals drawn at random from the same population compared to individuals drawn at random from populations in the same cluster. Significance levels were estimated by comparing Phi_{PT} values to a distribution of values obtained from 999 permutations assigning individuals randomly to populations of the same size using a Monte Carlo procedure.

To test whether metal exposure resulted in a stronger differentiation at particular loci in the genome, an outlier loci analysis was performed with the programs Bayescan (Foll & Gaggiotti 2008) and Spatial Analysis Method (Joost *et al.* 2008). Bayescan identifies candidate loci under natural selection from genetic data based on differences in allele frequencies between populations (Foll & Gaggiotti 2008). It applies a Bayesian hierarchical method based on the multinomial Dirichlet model calculating F_{ST} per locus and population and is suited for dominant markers like AFLPs. Selection is introduced by decomposing locus–population F_{ST} coefficients into a population-specific component (beta), shared by all loci and a locus-specific component (alpha) shared by all the populations using a logistic regression. Departure from neutrality at a given locus is assumed when the locus-specific component is necessary to explain the observed pattern of diversity (i.e. when alpha is significantly different from 0). This leads to

two alternative models for each locus, including the alpha component to model selection or leaving it out. BayeScan implements a reversible-jump MCMC algorithm to estimate the posterior probability of each one of these models. For each locus, BayeScan calculates a posterior probability for the model including selection. A Bayes factor of 3 corresponding to a posterior probability of 0.76, is already considered as being a "substantial" evidence for selection. Selection of a locus as outlier is based on the posterior probability and model choice on the Bayes Factor. Table 1 relates the Bayes Factor to probabilities and describes the strength of the evidence for selection for different levels.

Posterior probability ($\alpha \neq 0$)	Bayes Factor (BF)	log10(BF)	Jeffreys' interpretation
0.50 - 0.76	1 – 3	0 - 0,5	barely worth mentioning
0.76 - 0.91	3 -10	0.5 - 1	Substantial
0.91 - 0.97	10 – 32	1 - 1.5	Strong
0.97 - 0.99	32 – 100	1.5 - 2	very strong
0.99 - 1.00	100 - ∞	2 - ∞	Decisive

Table 1 | Relation between the Bayes Factor and probabilities and the strength of the evidence for selection for different levels.

For dominant markers, Bayescan appears better suited than other known applications like DetSel and DFdist with a higher detection of truly selective loci, least false positives and automatic optimization of parameters (Beaumont & Balding 2004; Manel *et al.* 2009; Perez-Figueroa *et al.* 2010). As the program calculates population-specific F_{ST} coefficients and hence allows for different demographic histories and different amounts of genetic drift between populations, it is robust against complex demographic scenarios for neutral differentiation. BayeScan incorporates the uncertainty on allele frequencies due to small sample sizes. Since it is a likelihood method and as shown in practice, very small sample size can be used (15 individuals per population as a guideluine, Foll & Gaggiotti 2008; Beaumont & Balding 2004), with the risk of a low power, but with no particular risk of bias. We defined following model parameters: sample size

(number of iterations) of 5000, thinning interval (number of iterations between two samples, reduces autocorrelation) of 50, 20 pilot runs (to determine proposal distibutions), pilot run length of 5000 iterations and an additional burn in of 50.000 (number of iterations before sampling). As suggested by Foll & Gaggiotti (2008), the prior odds value was set to 10 to correct for multiple testing.

Spatial Analysis Method (*SAM)* is a Windows program designed to detect candidate loci for selection in whole-genome scans. It also gives valuable clues regarding the ecological factors responsible for the selection process (Joost *et al.* 2008). The method used is based on multiple univariate logistic regression models to test for associations between allelic frequencies at marker loci and environmental variables. The software reads matrices constituted of presence/absence of molecular markers, and of the corresponding environmental parameters at sampling locations. To ensure the robustness of the method, two statistical tests (likelihood ratio G, and Wald) assess the significance of coefficients calculated by the logistic regression function at a significance threshold of both 95% and 99%. A model is considered significant only if both tests reject the null hypothesis of no relationship (Joost *et al.* 2007). Because multiple hypothesis testing is involved, Bonferroni correction was applied. SAM applies a conservative Bonferroni correction by dividing considering the correlation significant when p< threshold p (0,001) divided through the numer of comparisons (Shaffer 1995). AFLP data are ideal for logistic regression because their distribution is binomial information. The individual is the reference unit in SAM, which thus functions independently of any notion of population. This is an advantage for small sample sizes and when using dominant markers such as AFLPs, for which the classical methods are dependent on theoretical models of population genetics (e.g. Hardy–Weinberg equilibrium) (Joost *et al.* 2007). We included Cd, Zn and Cu concentration (µg/g ww) as environmental variables and longitude and latitude of the location as geographic information.

3 RESULTS

Overall, a mean number of 185 (96.4%) segregating bands and 117 bands per individual were observed (AFLPSurv) out of 192 markers. Only populations CM-P and CH-P1 showed one private marker each.

Comparing diversity revealed rather large differences among the six populations. Relative percentages of polymorphic loci (PLP, AFLPSurv) ranged between 84% and 97%, with the highest and lowest percentages observed in contaminated populations CM-P and CH-P2, respectively. Levels of expected heterozygosity (Hj, AFLPSurv) ranged between 0.34 and 0.40, with the lowest values present in the polluted populations CH-P1 and CH-P2) respectively, and the highest value in reference population CH-R2 (*Table 2*). Population CM-P showed a high diversity compared to the other polluted populations, while CH-R1 showed a low diversity compared to the other reference populations. None of the two genetic diversity indices correlated significantly with the average metal content in the spiders (*Table 3*).

Pop	[Cd] (µg/g ww)	[Zn] (µg/g ww)	N	#loc_P	PLP	Hj	SE (Hj)
CH-R1	4.05	85.6	8	176	91.7%	0.34	0.0099
CH-R2	3.76	108	9	182	94.8%	0.40	0.0089
CM-R	1.8	96.1	11	184	95.8%	0.37	0.0095
CH-P2	8.71	158	7	161	83.9%	0.35	0.011
CH-P1	20.91	164	14	175	91.1%	0.34	0.011
CM-P	3.29	134	17	187	97.4%	0.38	0.0093
Average				177.5	92.45%	0.36	0.0100

Table 2 | Genetic diversity within the six populations inferred from the percentage of polymorphic loci (PLP) and expected heterozygosity (Hj). N= sample size; #loc_P= number of polymorphic loci.

	PLP		Hj	
	r_P	p	r_P	p
[Cd]	-0.43	0.39	-0.56	0.23
[Zn]	-0.5	0.31	-0.29	0.58

Table 3 | Correlation (Pearson; r_P) between two genetic diversity measures (percentage of polymorphic loci (PLP) and expected heterozygosity (Hj) and metal concentrations of Cd and Zn in six P. saltans populations.

The average level of genetic differentiation (F_{ST}) among the six populations was 0.0795, which was significantly higher than expected under a random distribution of alleles among populations (-0.0163;0.0152, 99% CL; AFLPSurv). Based on pairwise F_{ST}, Nei GD (AFLPSurv) and Phi_{PT} (AMOVA) values (*Table 4,* with values significant after Bonferroni correctionindicated by a *), populations CH-P2/CH-P1 versus CM-R/CH-R1 were most strongly differentiated. In contrast, CM-P was only weakly differentiated from the other populations, most strongly so from CH-R1.

	CH-P1			CH-P2			CM-P		
	F_{ST}	NeiGD	Phi_{PT}	F_{ST}	NeiGD	Phi_{PT}	F_{ST}	NeiGD	Phi_{PT}
CH-P1	0.000	0.000	0.000	0.068	0.039	0.001*	0.030	0.017	0.002*
CH-P2	0.068	0.039	0.121	0.000	0.000	0.000	0.036	0.021	0.005
CM-P	0.030	0.012	0.083	0.036	0.021	0.072	0.000	0.000	0.000
CH-R1	0.164	0.107	0.253	0.157	0.103	0.261	0.074	0.046	0.075
CH-R2	0.071	0.045	0.001	0.024	0.014	0.065	0.003	0.002	0.013
CM-R	0.169	0.118	0.267	0.161	0.113	0.273	0.067	0.044	0.082

Table 4 | Genetic differentiation between six P. saltans populations. Pairwise F_{ST}, Nei' genetic distance and Phi_{PT} values are given for all pairwise comparisons. For Phi_{PT}, values above the diagonal represent p-values and are indicated with a start is still significant after Bonferroni coorection (p<0.0033). Values in red refer to pairwise comparisons between polluted populations, values in green refer to pairwise comparisons between reference populations, values in yellow refer to pairwise comparisons between polluted and reference populations.

	CH-R1			CH-R2			CM-R		
	F_{ST}	NeiGD	Phi_{PT}	F_{ST}	NeiGD	Phi_{PT}	F_{ST}	NeiGD	Phi_{PT}
CH-P1	0.164	0.107	0.001*	0.071	0.045	0.001*	0.169	0.118	0.001*
CH-P2	0.157	0.103	0.001*	0.024	0.014	0.004	0.161	0.113	0.001*
CM-P	0.074	0.046	0.003*	0.003	0.002	0.202	0.067	0.044	0.001*
CH-R1	0.000	0.000	0.000	0.067	0.042	0.005	0.007	0.004	0.039
CH-R2	0.067	0.042	0.093	0.000	0.000	0.000	0.062	0.042	0.001*
CM-R	0.007	0.004	0.026	0.062	0.042	0.112	0.000	0.000	0.000

Table 4 continued | Genetic differentiation between six P. saltans populations. Pairwise F_{ST}, Nei' genetic distance and Phi_{PT} values are given for all pairwise comparisons. For Phi_{PT}, values above the diagonal represent p-values and are indicated with a start is still significant after Bonferroni coorection (p<0.0033). Values in red refer to pairwise comparisons between polluted populations, values in green refer to pairwise comparisons between reference populations, values in yellow refer to pairwise comparisons between polluted and reference populations.

When grouping populations according to pollution level, a significant degree of differentiation among clusters was observed, that accounted for 7% of the total variation (Phi_{RT}=0.075; p=0.001), and was of a comparable magnitude to the differentiation among populations within groups (Phi_{PR}= 0.084; p=0.001). In contrast, when the two geographic clusters where set as regions no significant differentiation among regions was observed (Phi_{RT}=-0.018; p=1). Outlier detection first was performed on three different data structures: (i) all six populations, (ii) polluted versus reference populations, and (iii) both geographic clusters. Next, Bayescan analysis was performed on all pairwise population comparisons, divided in three different groups (i.e. within and between polluted and reference populations). When setting $\log_{10}$ BF=0.5 (substantial evidence for selection) as a detection minimum, 13 outlier loci (6.78%) were detected, out of which 4 (L9, L186, L160 and L181; 2,08%) were possibly linked to metal pollution since they were detected in comparisons between polluted and reference populations only. None of these markers appeared as outlier

in all pairwise combinations between a polluted and a reference population. Two of them (L9 and L186) showed a decisive evidence for selection ($\log_{10}$BF>2) (*Table 5*).

SAM analysis quantified the extent to which outlier loci were associated with the degree of metal exposure measured as average Cd and Zn body burden in each population. With a significance level of 95% after Bonferroni correction ($p < 4.34\ 10^{-5}$), a total of 25 markers (13%) were significantly related to Cd concentration, and 8 markers (4%) were significantly related to Zn for both G and Wald tests (*Appendix 1*). Five of the markers correlated to Zn and one related to Cd concentration were also detected as outliers in Bayescan(*Table 5*). Ten markers that were only detected by SAM appeared to be fixed in two or more reference populations. Four markers detected by Bayescan (of which two were also detected by SAM) were fixed in two reference populations. With a significance level of 99% after Bonferroni correction ($p < 8.86\ 10^{-6}$), eight markers (4%) were significantly related to Cd concentration, and 2 markers (1%) were significantly related to Zn for both G and Wald tests. Of these Cd related markers, one marker only (L186) was detected by Bayescan as possibly related to pollution. From allele frequencies (AFLP-SURV) marker L186 appeared to be fixated in 2 reference populations and absent in 2 polluted populations. In contrast, none of the two markers related to Zn concentration were detected by Bayescan. None of the markers is detected as an outlier when geographic location was set as a variable in SAM.

Locus	log10(BF)			Number of pairwise comparisons			Cd /Zn	Con-clusion
	Total	Pol vs Ref	Geo-graphic	pol-ref	ref-ref	pol-pol		
L9	3.70****	0.77*	0.13	4	0	0	Zn	metal-linked
L12	1.11**	0.01	0.19	0	1	0	/	false positive
L29	2.43****	0.07	0.047	2	2	0	/	
L49	1.06**	-0.01	0.33	0	0	1	/	
L50	0.49*	-0.09	0.76	1 (CH-P1)	0	1 (CH-P1)	/	population specific
L58	1.09**	0.75*	0.006	0	0	0	/	
L59	1.2751**	-0.018	-0.026	1 (CH-R1)	1 (CH-R1)	0	Zn	false positive or pop specific
L139	2.44****	0.23	0.23	2 (CH-P1)	0	0	Zn	pop specific
L149	1.07**	0.59*	-0.02	0	0	0	/	
L152	0.53*	0.83**	-0.03	0	0	0	Zn	
L160	1.93***	1.05**	-0.05	2 (CH-P1)	0	0	/	metal-linked
L181	0.94*	1.09**	-0.04	1	0	0	Cd	metal-linked
L186	∞****	0.57*	0.26	4	0	1	Zn	metal-linked
Total	13	7	0					

*Table 5 | Summary of outliers detected in Bayescan with a log_{10} BF of at least 0.50 in one of the datasets. Outlier detection was performed by structuring the populations according to (i) six individual populations (Total), (ii) polluted versus reference populations (Pol vs Ref) and (iii) two geographic clusters (Geographical). Afterwards an outlier loci detection was performed on all pairwise comparisons and the number of times the locus was detected as an outlier is given. * Reflect Jeffrey's interpretation (table 1) with **** indicating decisive, *** very strong, ** strong and * substantial evidence. Cd/Zn indicates whether the locus was found as an outlier in SAM linked to Cd or Zn concentration.*

4 DISCUSSION

Exposure to stressors often results in strong selection for tolerance traits which may result in a correlated response of fitness traits that are genetically correlated with stress resistance (Pérez and Garcia 2002; Van Straalen & Timmermans 2002; Merila *et al.* 2001; Hendrickx *et al.* 2008).

In the present study, we addressed how exposure to metal pollution may alter allele frequency distributions at a genome wide scale. First, we tested if exposure to these stressors resulted in an overall decrease of genetic variation, probably reflecting strong selection. We base our discussion on the measure of expected heterozygosity (Hj) since it is less sensitive to the differences in sample sizes in our dataset (Pruett & Winker 2008; Frankham *et al.* 2010). However, no direct evidence for such genetic erosion was present in the studied populations although for expected heterozygosity (Hj) the highest diversity was detected in a reference population (CH-R2) and the lowest diversity was present in a polluted population (CH-P1). Such limited effect of metal pollution on diversity might be due to an overarching effect of other demographic processes related to the biology of the species. The preferred habitat of *P. saltans* are forest clearings, which are generally of a transient nature. The very high metal concentrations at the polluted sites however did not permit forest regrowth and persisted for several decades. Hence, *P. saltans* populations at polluted sites are often much older and therefore characterized by a longer term influx of migrants and higher genetic diversity that could counteract potential diversity reducing effects from strong selection regimes. Thus, reference sites in our study might be considered to be subjected more strongly to accidental founder events and thus reducing genetic diversity. Likewise, in reviews by Van Straalen & Timmermans (2002) and Williams & Oleksiak (2008) and Dibattista (2008). several studies did not show significant reductions in genetic diversity in populations exposed to different kinds of toxicants using different genetic markers. This can also be explained by a steady influx of migrants and resulting gene flow since the distance between the populations was small and effects of pollution were relatively small. However, in this study, we did not estimate the degree of gene flow between the different populations, as our data show that a main

assumption for models that estimate migration rates, i.e. selective neutrality of the markers, is likely to be violated.

Second, we compared the degree of genetic differentiation between differentially exposed populations to the differentiation between populations that were not differentially exposed. The largest degree of genetic differentiation was observed between polluted and reference populations. Given that metal pollution was not related to the spatial structuring of the sampled populations, this result indicates that gene flow between differentially exposed populations was at least partially restricted. The two underlying mechanisms that can result in such a pattern are (i) very strong selection against maladapted individuals, which would impede gene flow among differentially adapted individuals or (ii) repeated selection of identical alleles in exposed populations resulting in a strong differentiation at a few loci among differentially exposed populations (i.e. presence of outlier loci). In the latter case, neutral genes would be expected to be freely exchanged among the differentially exposed populations. Results from both Bayescan and SAM models indeed suggested that identical alleles were selected in polluted versus reference populations and were involved in adaptation to pollution. Two of the markers detected as outliers possibly related to metal pollution in Bayescan (L9 and L186) also appeared to be significantly related to Zn concentration in the SAM analysis. The SAM analysis showed much more outliers, especially related to Cd concentration. All four outliers detected by Bayescan as potentially related to metal pollution were also related to Zn concentration. When the significance level was raised to 0.01, SAM detected far less outliers (10) and only one (L186), related to Zn concentration also was detected by Bayescan. Given that the same loci were detected with two methods that differ profoundly in their approach (regression versus group comparison) and algorithms (likelihood based versus Bayesian), we believe that the loci were indeed true outliers correlated with metal pollution. Since multiple population comparisons were performed, including populations from the two geographic clusters, and outliers also occurring in comparisons among reference populations were excluded, the outliers detected in Bayescan are unlikely to be false. Outlier behavior is most likely related to metal contamination as indicated by the SAM analysis. The percentage of outlier

loci detected by Bayescan (6.68% in toal) agrees well with other studies (reviewed in Nosil *et al.* 2009) while the 2,08% related to metal is rather low. The percentage detected by SAM (17%, correlated to Zn or Cd) is very high compared to other studies, and this merits further study.

A disadvantage of using AFLP markers, besides their dominance, is that the applied protocol does not allow to sequence the fragments that exhibit signatures of diversifying selection (Bensch & Akesson 2005). This renders it impossible to potentially identify the genes involved in adaptation and, hence, to translate these results into a functional context. With the advancement of next-generation sequencing techniques, a genotype-by-sequencing (GBS) or Restriction site Associated DNA (RAD) sequencing approach, wherein all amplified fragments are sequenced directly rather than assessing fragment length variation, would be an extremely valuable tool to provide more information on the genes involved in adaptation to this severe and persistent environmental stressor.

Conclusion

The results from these analyses thus suggest that exposure to metal pollution in this metapopulation selects identical alleles in the face of homogenizing gene flow thereby maintaining genetic variation and genetic differentiation indicative of local adaptation.

Author's contribution

Debbie Eraly and Frederik Hendrickx conceived and designed the study and wrote the manuscript together with Luc Lens. Debbie Eraly prepared the samples for AFLP analysis and scored the gels under supervision of Peter Breyne, who also provided theoretical input and optimized the protocol. Debbie Eraly and Frederik Hendrickx analyzed the data.

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Appendix 1 - Results SAM and Bayescan analysis AFLP data *Pradosa saltans*

SAM		Marker3			Marker9			Marker12			Marker21			Marker29		
G test		**G**	**P (G)**		**G**	**P (G)**		**G**	**P (G)**		**G**	**P (G)**		**G**	**P (G)**	
p=0,05	long	2.54	1.11E-01		4.52	3.35E-02		4.37	3.65E-02		9.80	1.74E-03		NaN	NaN	
	lat	6.25	1.24E-02		8.07	4.50E-03		4.00	4.54E-02		21.18	4.17E-06	***	NaN	NaN	
	Cd	27.12	1.91E-07	***	18.27	1.92E-05	***	0.07	7.87E-01		38.85	4.57E-10	***	NaN	NaN	
	Zn	23.97	9.76E-07	***	21.61	3.35E-06	***	1.99	1.59E-01		24.06	9.32E-07	***	NaN	NaN	
	Cu	5.26	2.18E-02		10.07	1.50E-03		5.40	2.01E-02		0.58	4.46E-01		NaN	NaN	
Wald test		**Wald b1**	**P (Wald b1)**		**Wald b1**	**P (Wald b1)**		**Wald b1**	**P (Wald b1)**		**Wald b1**	**P (Wald b1)**		**Wald b1**	**P (Wald b1)**	
p=0,05	long	1.54	6.15E-02		2.09	1.84E-02		2.05	2.02E-02		2.51	6.06E-03		2.19	1.41E-02	
	lat	2.30	1.07E-02		2.75	2.96E-03		1.97	2.46E-02		1.77	3.85E-02		2.46	6.94E-03	
	Cd	-4.30	8.69E-06	***	-2.91	1.80E-03		0.27	3.94E-01		-4.73	1.13E-06	***	-1.34	9.04E-02	
	Zn	-3.87	5.41E-05		-4.06	2.43E-05	***	-1.39	8.16E-02		-3.43	3.07E-04		-2.99	1.39E-03	
	Cu	2.16	1.55E-02		2.96	1.55E-03		2.24	1.27E-02		0.75	2.26E-01		3.06	1.11E-03	
Cumulated G and W test for Cd or Zn (p=0.01)											Cd					
Bayescan																
log10(BF) in nr pairwise comparisons conclusion	Total	< substantial			3.70 ****			1.11 **			< substantial			2.43 ****		
	Pol vs Ref	< substantial			0.77 *			0.01			< substantial			0.07		
	Geogr	< substantial			0.13			0.19			< substantial			0.047		
	pol-ref	< substantial			4			0			< substantial			2		
	ref-ref	< substantial			0			1			< substantial			2		
	pol-pol	< substantial			0			0			< substantial			0		
		no outlier			metal-linked			false positive			no outlier					
Allele freq	Total	0.83			0.44			0.36			0.95			0.50		
	CH-P2	0.71			0.00			0.00			0.86			0.00		
	CH-P3	0.79			0.00			0.07			0.93			0.29		
	CM-P	0.76			0.41			0.35			1.00			0.47		
	CH-R1	1.00			0.88			0.75			1.00			1.00		
	CH-R2	0.78			0.44			0.11			0.89			0.14		
	CM-R	1.00			1.00			0.91			1.00			1.00		

Appendix 1 - Results SAM and Bayescan analysis AFLP data *Pradosa saltans*

SAM		Marker37			Marker49			Marker50			Marker55		
G test		**G**	**P (G)**		**G**	**P (G)**		**G**	**P (G)**		**G**	**P (G)**	
p=0,05	long	6.32	1.19E-02		3.26	7.09E-02		3.15	7.60E-02		7.81	5.19E-03	
	lat	14.54	1.37E-04		3.06	8.01E-02		1.79	1.81E-01		13.44	2.47E-04	
	Cd	45.39	1.61E-11	***	0.16	6.86E-01		5.23	2.22E-02		22.36	2.26E-06	***
	Zn	28.05	1.18E-07	***	0.95	3.31E-01		0.13	7.19E-01		14.15	1.69E-04	
	Cu	0.66	4.17E-01		1.21	2.71E-01		1.82	1.78E-01		0.49	4.86E-01	
Wald test		**Wald b1**	**P (Wald b1)**		**Wald b1**	**P (Wald b1)**		**Wald b1**	**P (Wald b1)**		**Wald b1**	**P (Wald b1)**	
p=0,05	long	2.24	1.25E-02		1.76	3.93E-02		1.74	4.05E-02		2.41	7.94E-03	
	lat	2.53	5.62E-03		1.71	4.40E-02		1.32	9.26E-02		2.58	4.94E-03	
	Cd	-4.57	2.44E-06	***	-0.41	3.43E-01		2.12	1.70E-02		-4.23	1.17E-05	***
	Zn	-3.55	1.90E-04		-0.97	1.67E-01		0.36	3.60E-01		-3.19	7.17E-04	
	Cu	0.80	2.11E-01		1.09	1.39E-01		1.33	9.20E-02		0.69	2.45E-01	
Cumulated G and W test for Cd or Zn (p=0.01)			Cd										
Bayescan													
log10(BF) in nr pairwise comparisons conclusion	Total		< substantial			1.06 **			0.49 *			< substantial	
	Pol vs Ref		< substantial			-0.01			-0.09			< substantial	
	Geogr		< substantial			0.33			0.76			< substantial	
	pol-ref		< substantial			0			1 (CH-P1)			< substantial	
	ref-ref		< substantial			0			0			< substantial	
	pol-pol		< substantial			1			1 (CH-P1)			< substantial	
			no outlier						population specific			no outlier	
Allele freq	Total		0.94			0.52			0.39			0.92	
	CH-P2		0.86			0.29			0.14			0.71	
	CH-P3		0.93			0.00			0.00			0.93	
	CM-P		0.94			0.65			0.53			0.94	
	CH-R1		0.88			0.75			0.50			1.00	
	CH-R2		1.00			0.56			0.33			0.89	
	CM-R		1.00			0.91			0.82			1.00	

Appendix 1 - Results SAM and Bayescan analysis AFLP data *Pradosa saltans*

SAM		Marker56			Marker58			Marker59			Marker64		
G test		**G**	**P (G)**		**G**	**P (G)**		**G**	**P (G)**		**G**	**P (G)**	
p=0,05	long	6.11	1.35E-02		3.61	5.73E-02		14.01	1.82E-04		6.41	1.13E-02	
	lat	9.73	1.82E-03		7.56	5.98E-03		21.20	4.15E-06	***	10.28	1.35E-03	
	Cd	22.38	2.24E-06	***	13.23	2.75E-04		25.88	3.63E-07	***	18.53	1.67E-05	***
	Zn	9.36	2.22E-03		17.29	3.21E-05	***	25.25	5.04E-07	***	10.06	1.52E-03	
	Cu	0.00	9.54E-01		4.72	2.99E-02		6.08	1.37E-02		0.22	6.40E-01	
Wald test		**Wald b1**	**P (Wald b1)**		**Wald b1**	**P (Wald b1)**		**Wald b1**	**P (Wald b1)**		**Wald b1**	**P (Wald b1)**	
p=0,05	long	2.16	1.55E-02		1.82	3.42E-02		-3.48	2.50E-04		2.19	1.41E-02	
	lat	2.38	8.66E-03		2.50	6.16E-03		-4.08	2.21E-05	***	2.39	8.49E-03	
	Cd	-4.23	1.19E-05	***	-3.41	3.20E-04		3.03	1.21E-03		-3.97	3.63E-05	***
	Zn	-2.71	3.36E-03		-3.59	1.68E-04		4.29	8.89E-06	***	-2.78	2.72E-03	
	Cu	0.06	4.77E-01		2.06	1.97E-02		-2.36	9.16E-03		0.47	3.21E-01	
Cumulated G and W test for Cd or Zn (p=0.01)													
Bayescan													
log10(BF) in nr pairwise comparisons conclusion	Total	< substantial			1.09**			1.2751**			< substantial		
	Pol vs Ref	< substantial			0.75 *			-0.018			< substantial		
	Geogr	< substantial			0.006			-0.026			< substantial		
	pol-ref	< substantial			0			1 (CH-R1)			< substantial		
	ref-ref	< substantial			0			1 (CH-R1)			< substantial		
	pol-pol	< substantial			0			0			< substantial		
		no outlier						false positive			no outlier		
Allele freq	Total	0.97			0.68			0.38			0.91		
	CH-P2	1.00			0.29			1.00			0.86		
	CH-P3	1.00			0.29			0.29			0.86		
	CM-P	1.00			0.71			0.35			0.94		
	CH-R1	1.00			1.00			0.00			0.88		
	CH-R2	0.89			0.89			0.78			0.89		
	CM-R	0.91			1.00			0.09			1.00		

Appendix 1 - Results SAM and Bayescan analysis AFLP data *Pradosa saltans*

SAM		Marker65			Marker75			Marker76			Marker78		
G test		**G**	**P (G)**		**G**	**P (G)**		**G**	**P (G)**		**G**	**P (G)**	
p=0,05	long	7.58	5.91E-03		7.48	6.24E-03		5.46	1.95E-02		2.15	1.42E-01	
	lat	19.10	1.24E-05	***	12.02	5.27E-04		10.59	1.14E-03		6.44	1.11E-02	
	Cd	44.90	2.07E-11	***	22.82	1.78E-06	***	33.11	8.73E-09	***	42.16	8.42E-11	***
	Zn	23.69	1.13E-06	***	12.45	4.17E-04		24.22	8.57E-07	***	32.03	1.52E-08	***
	Cu	0.04	8.37E-01		0.35	5.53E-01		3.57	5.89E-02		5.21	2.24E-02	
Wald test		**Wald b1**	**P (Wald b1)**		**Wald b1**	**P (Wald b1)**		**Wald b1**	**P (Wald b1)**		**Wald b1**	**P (Wald b1)**	
p=0,05	long	2.26	1.20E-02		2.46	6.92E-03		2.19	1.42E-02		1.43	7.69E-02	
	lat	1.88	2.99E-02		2.83	2.35E-03		2.83	2.35E-03		2.33	9.90E-03	
	Cd	-4.39	5.61E-06	***	-4.18	1.46E-05	***	-4.24	1.10E-05	***	-4.02	2.94E-05	***
	Zn	-3.11	9.22E-04		-3.15	8.20E-04		-3.94	4.13E-05		-4.02	2.87E-05	***
	Cu	0.21	4.19E-01		0.59	2.78E-01		1.81	3.50E-02		2.15	1.59E-02	
Cumulated G and W test for Cd or Zn (p=0.01)			Cd										
Bayescan													
log10(BF) in nr pairwise comparison conclusion	Total		< substantial			< substantial			< substantial			substantial	
	Pol vs Ref		< substantial			< substantial			< substantial			< substantial	
	Geogr		< substantial			< substantial			< substantial			< substantial	
	pol-ref		< substantial			< substantial			< substantial			< substantial	
	ref-ref		< substantial			< substantial			< substantial			< substantial	
	pol-pol		< substantial			< substantial			< substantial			< substantial	
			no outlier			no outlier			no outlier			no outlier	
Allele freq	Total		0.92			0.77			0.83			0.73	
	CH-P2		1.00			0.71			0.71			0.86	
	CH-P3		0.71			0.43			0.79			0.21	
	CM-P		1.00			0.88			0.71			0.71	
	CH-R1		0.88			0.75			1.00			1.00	
	CH-R2		1.00			0.89			0.89			0.89	
	CM-R		1.00			1.00			1.00			1.00	

Appendix 1 - Results SAM and Bayescan analysis AFLP data *Pradosa saltans*

SAM		Marker87			Marker99			Marker102		Marker103		
G test		**G**	**P (G)**		**G**	**P (G)**		**G**	**P (G)**	**G**	**P (G)**	
p=0,05	long	2.07	1.50E-01		1.65	1.99E-01		0.66	4.16E-01	6.89	8.68E-03	
	lat	5.17	2.30E-02		4.16	4.13E-02		0.94	3.33E-01	13.79	2.05E-04	
	Cd	24.16	8.86E-07	***	19.94	8.00E-06	***	3.67	5.53E-02	28.67	8.60E-08	***
	Zn	10.66	1.10E-03		8.53	3.50E-03		1.82	1.78E-01	18.08	2.12E-05	***
	Cu	0.02	8.97E-01		0.04	8.44E-01		0.29	5.92E-01	0.32	5.72E-01	
Wald test		**Wald b1**	**P (Wald b1)**		**Wald b1**	**P (Wald b1)**		**Wald b1**	**P (Wald b1)**	**Wald b1**	**P (Wald b1)**	
p=0,05	long	1.37	8.53E-02		1.23	1.09E-01		0.787345229	2.16E-01	2.312080007	1.04E-02	
	lat	1.99	2.33E-02		1.82	3.48E-02		0.925077982	1.77E-01	2.568872159	5.10E-03	
	Cd	-4.29	8.96E-06	***	-4.01	3.04E-05	***	-1.949255247	2.56E-02	-4.52753392	2.98E-06	***
	Zn	-2.79	2.61E-03		-2.56	5.30E-03		-1.295341832	9.76E-02	-3.398647409	3.39E-04	
	Cu	-0.13	4.48E-01		-0.20	4.22E-01		0.529724752	2.98E-01	0.561528124	2.87E-01	
Cumulated G and W test for Cd or Zn (p=0.01)											Cd	
Bayescan												
log10(BF)	Total		< substantial			< substantial			substantial		< substantial	
	Pol vs Ref		< substantial			< substantial			< substantial		< substantial	
	Geogr		< substantial			< substantial			< substantial		< substantial	
in nr	pol-ref		< substantial			< substantial			< substantial		< substantial	
pairwise	ref-ref		< substantial			< substantial			< substantial		< substantial	
comparisons	pol-pol		< substantial			< substantial			< substantial		< substantial	
conclusion			no outlier			no outlier			no outlier		no outlier	
Allele	Total		0.94			0.95			0.82		0.94	
freq	CH-P2		1.00			1.00			1.00		0.71	
	CH-P3		1.00			1.00			0.36		1.00	
	CM-P		0.94			1.00			0.88		1.00	
	CH-R1		1.00			1.00			1.00		1.00	
	CH-R2		0.89			0.89			0.89		0.78	
	CM-R		0.82			0.82			1.00		1.00	

Appendix 1 - Results SAM and Bayescan analysis AFLP data *Pradosa saltans*

SAM		Marker105			Marker111			Marker122		
G test		**G**	**P (G)**		**G**	**P (G)**		**G**	**P (G)**	
p=0,05	long	2.28	1.31E-01		1.56	2.12E-01		6.16	1.31E-02	
	lat	5.86	1.55E-02		4.39	3.62E-02		14.03	1.79E-04	
	Cd	30.53	3.29E-08	***	22.18	2.48E-06	***	54.11	1.90E-13	***
	Zn	13.96	1.86E-04		19.54	9.87E-06	***	32.86	9.91E-09	***
	Cu	0.05	8.30E-01		3.57	5.89E-02		1.23	2.68E-01	
Wald test		**Wald b1**	**P (Wald b1)**		**Wald b1**	**P (Wald b1)**		**Wald b1**	**P (Wald b1)**	
p=0,05	long	1.467203589	7.12E-02		1.209695287	1.13E-01		2.220387437	1.32E-02	
	lat	2.240676679	1.25E-02		1.915451456	2.77E-02		2.559305734	5.24E-03	
	Cd	-4.352928969	6.72E-06	***	-4.223280368	1.20E-05	***	-3.927331761	4.29E-05	
	Zn	-3.307129561	4.71E-04		-3.367654833	3.79E-04		-3.492786716	2.39E-04	
	Cu	0.214687168	4.15E-01		1.784776513	3.71E-02		1.084998663	1.39E-01	
Cumulated G and W test for Cd or Zn (p=0.01)		Cd								
Bayescan										
log10(BF) in nr pairwise comparisons conclusion	Total	< substantial			< substantial			< substantial		
	Pol vs Ref	< substantial			< substantial			< substantial		
	Geogr	< substantial			< substantial			< substantial		
	pol-ref	< substantial			< substantial			< substantial		
	ref-ref	< substantial			< substantial			< substantial		
	pol-pol	< substantial			< substantial			< substantial		
		no outlier			no outlier			no outlier		
Allele freq	Total	0.88			0.94			0.98		
	CH-P2	1.00			0.86			1.00		
	CH-P3	0.71			0.93			1.00		
	CM-P	0.94			0.88			0.94		
	CH-R1	1.00			1.00			1.00		
	CH-R2	0.89			1.00			1.00		
	CM-R	0.82			1.00			1.00		

Appendix 1 - Results SAM and Bayescan analysis AFLP data *Pradosa saltans*

SAM		Marker139			Marker141			Marker149			Marker152		
G test		**G**	**P (G)**		**G**	**P (G)**		**G**	**P (G)**		**G**	**P (G)**	
p=0,05	long	10.71	1.07E-03		1.56	2.11E-01		0.06	8.05E-01		3.55	5.96E-02	
	lat	16.64	4.53E-05		3.98	4.60E-02		0.83	3.63E-01		10.88	9.73E-04	
	Cd	32.34	1.29E-08	***	26.48	2.66E-07	***	13.41	2.50E-04		49.65	1.84E-12	***
	Zn	25.15	5.31E-07	***	15.73	7.31E-05		13.07	3.01E-04		45.50	1.53E-11	***
	Cu	7.22	7.23E-03		1.70	1.93E-01		3.51	6.09E-02		5.60	1.79E-02	
Wald test		**Wald b1**	**P (Wald b1)**		**Wald b1**	**P (Wald b1)**		**Wald b1**	**P (Wald b1)**		**Wald b1**	**P (Wald b1)**	
p=0,05	long	3.133452804	8.64E-04		-1.22	1.11E-01		0.25	4.03E-01		1.80	3.58E-02	
	lat	3.806321702	7.05E-05		-1.88	3.03E-02		0.88	1.89E-01		2.81	2.48E-03	
	Cd	-2.751988779	2.96E-03		4.38	5.89E-06	***	-3.42	3.08E-04		-3.37	3.79E-04	
	Zn	-4.256345832	1.04E-05	***	3.38	3.65E-04		-2.69	3.56E-03		-3.99	3.25E-05	***
	Cu	2.55896368	5.25E-03		-1.27	1.02E-01		1.73	4.20E-02		2.22	1.33E-02	
Cumulated G and W test for Cd or Zn (p=0.01)						Cd							
Bayescan													
log10(BF)	Total		2.44 ****			< substantial			1.07 **			0.53 *	
	Pol vs Ref		0.23			< substantial			0.59 *			0.83 **	
	Geogr		0.23			< substantial			-0.02			-0.03	
in nr	pol-ref		2 (CH-P1)			< substantial			0			0	
pairwise	ref-ref		0			< substantial			0			0	
comparisons	pol-pol		0			< substantial			0			0	
conclusion			population specific			no outlier							
Allele	Total		0.42			0.09			0.80			0.77	
freq	CH-P2		0.00			0.00			1.00			0.57	
	CH-P3		0.00			0.00			0.29			0.36	
	CM-P		0.47			0.29			0.81			0.81	
	CH-R1		0.88			0.00			1.00			1.00	
	CH-R2		0.33			0.11			1.00			1.00	
	CM-R		0.91			0.00			1.00			1.00	

Appendix 1 - Results SAM and Bayescan analysis AFLP data *Pradosa saltans*

SAM		Marker156			Marker158			Marker160			Marker162		
G test		**G**	**P (G)**		**G**	**P (G)**		**G**	**P (G)**		**G**	**P (G)**	
p=0,05	long	4.77	2.89E-02		3.70	5.43E-02		3.97	4.62E-02		1.54	2.15E-01	
	lat	11.59	6.63E-04		7.47	6.26E-03		7.40	6.52E-03		4.39	3.62E-02	
	Cd	48.08	4.09E-12	***	22.02	2.70E-06	***	15.66	7.57E-05		36.73	1.36E-09	***
	Zn	28.20	1.09E-07	***	9.73	1.81E-03		18.73	1.51E-05	***	20.42	6.21E-06	***
	Cu	0.89	3.44E-01		0.03	8.65E-01		7.17	7.43E-03		2.35	1.25E-01	
Wald test		**Wald b1**	**P (Wald b1)**		**Wald b1**	**P (Wald b1)**		**Wald b1**	**P (Wald b1)**		**Wald b1**	**P (Wald b1)**	
p=0,05	long	-2.04	2.09E-02		1.79	3.65E-02		1.94	2.65E-02		-1.22	1.12E-01	
	lat	-2.74	3.04E-03		2.32	1.03E-02		2.57	5.09E-03		-1.99	2.35E-02	
	Cd	3.98	3.47E-05	***	-4.21	1.26E-05	***	-3.46	2.74E-04		4.14	1.71E-05	***
	Zn	3.80	7.32E-05		-2.78	2.74E-03		-3.82	6.66E-05		3.77	8.09E-05	
	Cu	-0.93	1.76E-01		-0.17	4.32E-01		2.51	6.00E-03		-1.49	6.82E-02	
Cumulated G and W test for Cd or Zn (p=0.01)													
Bayescan													
log10(BF) in nr pairwise comparisons conclusion	Total	< substantial			< substantial			1.93 ***			< substantial		
	Pol vs Ref	< substantial			< substantial			1.05 **			< substantial		
	Geogr	< substantial			< substantial			-0.05			< substantial		
	pol-ref	< substantial			< substantial			2 (CH-P1)			< substantial		
	ref-ref	< substantial			< substantial			0			< substantial		
	pol-pol	< substantial			< substantial			0			< substantial		
	conclusion	no outlier			no outlier			metal-linked			no outlier		
Allele freq	Total	0.09			0.91			0.56			0.17		
	CH-P2	0.00			0.86			0.29			0.00		
	CH-P3	0.14			0.79			0.07			0.29		
	CM-P	0.12			0.94			0.53			0.35		
	CH-R1	0.00			0.88			1.00			0.13		
	CH-R2	0.11			1.00			0.78			0.00		
	CM-R	0.11			1.00			1.00			0.00		

Appendix 1 - Results SAM and Bayescan analysis AFLP data *Pradosa saltans*

SAM		Marker163			Marker169			Marker171			Marker176		
G test		**G**	**P (G)**		**G**	**P (G)**		**G**	**P (G)**		**G**	**P (G)**	
p=0,05	long	4.54	3.31E-02		10.51	1.18E-03		8.58	3.39E-03		3.65	5.62E-02	
	lat	12.39	4.31E-04		18.47	1.72E-05	***	16.86	4.01E-05	***	8.12	4.37E-03	
	Cd	35.50	2.55E-09	***	34.23	4.91E-09	***	40.31	2.16E-10	***	41.46	1.20E-10	***
	Zn	34.70	3.84E-09	***	26.50	2.64E-07	***	31.70	1.80E-08	***	19.21	1.17E-05	***
	Cu	2.17	1.40E-01		3.20	7.35E-02		4.62	3.16E-02		0.39	5.31E-01	
Wald test		**Wald b1**	**P (Wald b1)**		**Wald b1**	**P (Wald b1)**		**Wald b1**	**P (Wald b1)**		**Wald b1**	**P (Wald b1)**	
p=0,05	long	1.93	2.66E-02		-2.90	1.87E-03		2.76	2.92E-03		1.82	3.46E-02	
	lat	2.34	9.74E-03		-3.31	4.61E-04		3.57	1.80E-04		2.52	5.82E-03	
	Cd	-4.68	1.47E-06	***	3.96	3.73E-05	***	-3.22	6.49E-04		-4.25	1.08E-05	***
	Zn	-2.84	2.23E-03		4.12	1.93E-05	***	-4.47	3.93E-06	***	-3.62	1.46E-04	
	Cu	1.42	7.81E-02		-1.73	4.18E-02		2.07	1.94E-02		0.62	2.67E-01	
Cumulated G and W test for Cd or Zn (p=0.01)		Cd											
Bayescan													
log10(BF) in nr pairwise comparisons conclusion	Total	< substantial			< substantial			substantial			< substantial		
	Pol vs Ref	< substantial			< substantial			< substantial			< substantial		
	Geogr	< substantial			< substantial			< substantial			< substantial		
	pol-ref	< substantial			< substantial			< substantial			< substantial		
	ref-ref	< substantial			< substantial			< substantial			< substantial		
	pol-pol	< substantial			< substantial			< substantial			< substantial		
		no outlier			no outlier			no outlier			no outlier		
Allele freq	Total	0.98			0.16			0.73			0.89		
	CH-P2	1.00			0.57			0.14			1.00		
	CH-P3	1.00			0.00			0.71			0.86		
	CM-P	0.94			0.13			0.81			0.88		
	CH-R1	1.00			0.00			1.00			0.86		
	CH-R2	1.00			0.44			0.56			0.89		
	CM-R	1.00			0.00			1.00			0.91		

Appendix 1 - Results SAM and Bayescan analysis AFLP data *Pradosa saltans*

SAM		Marker180		Marker183			Marker186			Marker187	
G test		**G**	**P (G)**	**G**	**P (G)**		**G**	**P (G)**		**G**	**P (G)**
p=0,05	long	0.10	7.54E-01	12.23	4.70E-04		9.16	2.47E-03		6.87	8.75E-03
	lat	0.17	6.80E-01	30.20	3.89E-08	***	15.27	9.34E-05		11.24	7.99E-04
	Cd	2.01	1.57E-01	44.76	2.23E-11	***	34.83	3.60E-09	***	27.56	1.52E-07
	Zn	0.66	4.15E-01	32.79	1.02E-08	***	33.50	7.14E-09	***	14.72	1.24E-04
	Cu	0.09	7.70E-01	1.31	2.52E-01		13.97	1.86E-04		0.66	4.15E-01
Wald test		**Wald b1**	**P (Wald b1)**	**Wald b1**	**P (Wald b1)**		**Wald b1**	**P (Wald b1)**		**Wald b1**	**P (Wald b1)**
p=0,05	long	0.31	3.77E-01	2.81	2.45E-03		2.91	1.78E-03		-2.28	1.13E-02
	lat	0.41	3.40E-01	2.58	4.89E-03		3.66	1.25E-04		-2.49	6.36E-03
	Cd	-1.36	8.67E-02	-4.25	1.09E-05	***	-2.80	2.56E-03		4.49	3.50E-06
	Zn	-0.81	2.09E-01	-3.81	7.04E-05		-4.60	2.12E-06	***	3.17	7.52E-04
	Cu	0.29	3.85E-01	1.12	1.31E-01		3.38	3.66E-04		-0.80	2.11E-01
Cumulated G and W test for Cd or Zn (p=0.01)							Cd				
Bayescan											
log10(BF)	Total	0.94 *		< substantial			1000****			< substantial	
	Pol vs Ref	1.09 **		< substantial			0.57 *			< substantial	
	Geogr	-0.04		< substantial			0.26			< substantial	
in nr	pol-ref	1		< substantial			4			< substantial	
pairwise	ref-ref	0		< substantial			0			< substantial	
comparisons	pol-pol	0		< substantial			1			< substantial	
conclusion		metal-linked		no outlier			metal-linked			no outlier	
Allele	Total	0.47		0.92			0.45			0.06	
freq	CH-P2	0.57		0.57			0.00			0.00	
	CH-P3	0.57		0.93			0.00			0.00	
	CM-P	0.38		1.00			0.47			0.12	
	CH-R1	0.43		1.00			1.00			0.00	
	CH-R2	0.56		0.89			0.33			0.22	
	CM-R	0.36		1.00			1.00			0.00	

Fat bottomed girls
you make
the rockin' world
go round

Queen

CHAPTER 4 | CONDITION-DEPENDENT MATE CHOICE AND ITS IMPLICATIONS FOR POPULATION DIFFERENTIATION IN THE WOLF SPIDER *PIRATA PIRATICUS*

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ABSTRACT

When populations face different environmental conditions, both local adaptation and phenotypic plasticity may cause inter-population divergence of behavioral or phenotypic properties on which mate choice is based. If sustained, this may result in genetic differentiation even in presence of extant gene flow. Condition-dependence of mate choice is one of the main mechanisms explaining these environmental effects. We tested whether experimental food stress affects mate choice in male and female *Pirata piraticus* spiders from one heavily polluted and one unpolluted reference population. Compared to control females, food-stressed females from the reference population showed a decreased probability of copulation and preferred smaller mates. Females from the polluted population, in contrast, did not show a significant response to food stress and showed size-assortative mating, most strongly under food stress. We explain these results in two complementary ways. First, spiders from populations that are not adapted to cope with stress may be less willing to mate when eggs are not fully matured. Second, food-deprived females may show a larger responsiveness towards smaller males because the latter resemble prey more and hungry females tend to attack moving objects more often. Results from this study support the prediction that variation in body condition, driven by local ecological factors, may affect mating behavior and may ultimately lead to population divergence in important life history traits such as body size.

Key words: size-assortative mating; population divergence; sexual selection; resource availability; heavy metals; reproductive isolation; phenotypic plasticity

1 INTRODUCTION

It has long been recognized that environmental variation can shape the evolution of mating strategies through multiple effects on individual phenotype and condition (Ortigosa and Rowe 2002; Hunt et al. 2005; Fisher and Rosenthal 2006). In populations that are exposed to different environmental conditions, differential sexual selection regimes may result in spatial variation in selection pressures through various mechanisms. First, adaptation to different environments may cause divergence of behavioral or phenotypic properties that are involved in mate choice. As such, differential sexual selection regimes may result as a by-product of natural selection (the "by-product mechanism" sensu Funk 1998; Vines and Schluter 2006). Such spatio-temporal variation in selection may promote the maintenance of interpopulation genetic variation, even in the absence of geographical barriers (Schluter 2001; Coyne and Orr 2004; Hey 2006; Niemiller et al. 2008; Nosil 2008; Rasanen and Hendry 2008). Second, environmental factors such as predation risk, sex ratio, population density or food availability may affect mating behavior through phenotypic plasticity (Jennions and Petrie 1997). As stated by West-Eberhard (2005) and Crispo (2008), phenotypic plasticity could be a potent mechanism to establish phenotypic divergence that could lead to assortative mating and thus precede genetically based reproductive isolation.

Studies that examined environmental effects on mate choice mainly dealt with signal reliability and courtship behavior from a male perspective (Andersson 1994; Parsons 1995; Jennions and Petrie 1997; Kotiaho et al. 1998; Hoefler et al. 2008). For example, various empirical studies provided compelling evidence for the conditional handicap model (Zahavi 1977), i.e. the prediction that sexual traits are reliable indicators of male quality due to their cost of production, and are thus condition-dependent (e.g. Johnstone 1995; Rowe and Houle 1996; Parri et al. 1997; Kotiaho 2000; Uetz et al. 2002; Ahtiainen et al. 2006). How, and to what degree, variation in female mate choice can affect both the strength and direction of sexual selection, however, remains less well understood, especially in invertebrates (Jennions and Petrie 1997; Ortigosa and Rowe 2002; Archard et al. 2006). Because the strength of female resistance to engage in

mating is likely to be influenced by her energetic condition (Ortigosa and Rowe 2002), environmental variation that directly or indirectly affects resource availability may affect the strength of sexual selection either by altering optimal mating rates, or by causing differential sexual selection of phenotypes, thereby reinforcing adaptive divergence (Vines and Schluter 2006). Female mate choice consists of two main components, each of which may be condition-dependent (Burley and Foster 2006; Fisher and Rosenthal 2006; Hebets et al. 2008; Tigreros and Switzer 2008; Wilder and Rypstra 2008). Female 'responsiveness' refers to female willingness to engage in a mating, while female 'preference' refers to the propensity to mate with certain phenotypes (Jennions and Petrie 1997; Hunt et al. 2005). Conditions leading to a decrease in female responsiveness may either weaken sexual selection, if the likelihood of random mating increases, or strengthen it, if the threshold for male attractiveness increases. Female preference can be expected to be weak when costs associated with mate choice are high, the probability of meeting high quality mates is low, or when females are in poor condition (Real 1990; Pomiankowski et al. 1991; Clark et al. 1997; Hingle et al. 2001; Hunt et al. 2005). Alternatively, under energetic stress, costs of mating increase and hence also the benefit of mating with the best possible partner (Rowe et al. 1994; Ortigosa and Rowe 2002; Fisher and Rosenthal 2006).

Body size, through its positive correlation with physiological condition and fitness, is considered an important cue for mate choice behavior in a variety of organisms (Crespi 1989; Andersson 1994; Arnqvist et al. 1996). Selection for larger females is often associated with higher fecundity or higher offspring quality, while selection for larger males generally reflects sexual selection or male-male competition (Andersson 1994; Blanckenhorn 2000). If both males and females prefer larger mates - and while competing for the latter, larger individuals are more successful - mate choice may result in size-assortative mating (Crespi 1989). Apart from active mate choice, selection for body size might also result from mate availability, the importance of size in overcoming female resistance, male-male competition, physical constraints in the mating apparatus, or loading constraints (Crespi 1989; Harari et al. 1999; Taborsky et al 2009). Irrespective of the mechanism underlying these patterns, variation in

environmental or population characteristics that results in consistent between-population differences in body size or the strength of assortative mating, may reinforce reproductive isolation (Crespi 1989).

One type of environmental variation that is recognized to impose strong selective pressures in natural populations of plants and animals, is pollution with heavy metals (Fox 1995; Posthuma and Vanstraalen 1993). The mechanism underlying this relationship is that physiological defense mechanisms consume energy that cannot be allocated to other life history processes (Calow 1991). In addition, pollution driven changes in absolute and relative species abundance (e.g. Read et al. 1998; Lock et al. 2003) are expected to result in an overall reduction in suitable prey availability, which furthers constrains the total energy budget. In a study on the wolf spider *Pirata piraticus*, Hendrickx et al. (2003) showed that populations inhabiting metal polluted sites exhibited life history characteristics that confirm the reduction in resource acquisition. Key life history traits such as reproductive output were negatively related with average metal body burden. A subsequent reciprocal crossing experiment (Hendrickx et al. 2008) confirmed that this observed life history divergence had a genetic basis, making this ecological model an ideal case to test if, and to what extent, environmentally-driven selection between habitats can result in reproductive isolation (Schluter 2001). Building on these studies, we randomly assigned male and female *P. piraticus* collected in one heavily polluted and one unpolluted (reference) site to either a food stress or a control treatment, and subsequently conducted within- and across-population mating trials. A food stress treatment best mimics the variation in resource acquisition observed in the field and can be applied with high precision, contrary to a treatment with heavy metals. Results from these experiments were used to address the following research questions: (i) does genetic divergence in life history traits leads to assortative mating (ii) does reduced resource acquisition affect female and male mate choice; (iii) does this impact differ between populations that are differentially adapted to resource acquisition and (iv) can condition dependence in mate choice ultimately promote adaptive divergence between populations?

2 MATERIAL AND METHODS

2.1 Study system and spider maintenance

Male and female *Pirata piraticus* (Araneae: Lycosidae) were collected in two populations in Flanders (Belgium): (i) Damvallei (53°03' N, 3°50' E), an unpolluted freshwater marsh henceforth referred to as the reference population (R); (ii) Galgenschoor (51°18' N, 4°18' E), a tidal marsh located along the river Scheldt, heavily polluted by nearby industrial activities, referred to as the polluted population (P). These populations were selected based on earlier studies where they were shown to be two extremes of a life-history trait and pollution gradient (Hendrickx et al. 2003). Under natural conditions, adult females produce one or two egg sacs (May-August) with larger females breeding earlier in the season and showing larger clutch volumes and masses (Hendrickx et al. 2003). In an earlier mating experiment, where females were presented with different males on subsequent days and copulation was not prevented, females almost consistently mated only once (in only 6% out of 150 mating trials a female re-mated after being fertilized) (D. Eraly, unpubl. data). Contrary to most other wolf spiders that are used for studies on courtship behavior (e.g. Kotiaho et al 1996; Hebets and Uetz 2000; Töpfer-Hofmann et al 2000), *P. piraticus* male courtship behavior is short, less conspicuous and does not include pronounced leg or abdomen movements, and they lack obvious secondary sexual traits.

During Feb-Mar 2006, 158 (population R) and 232 (population P) spiders were collected, while an additional 152 spiders (population R) were collected in Feb 2007. To ensure virginity, all spiders were caught as subadults, i.e. prior to their last molt. They were housed individually in plaster-layered vials under optimal growth conditions (dark-light regime of 16h:8h, temperature of 20°C) and fed with flight-deficient *Drosophila melanogaster* reared on a dog food-banana-oat medium (Mayntz and Toft 2001). Once captured, individuals of each population were randomly assigned to either a food-stress treatment (S) or a control treatment (C). Every third day, subadults of the S-treatment received 2 flies, adult males 1 fly and adult females 6 flies. Under the C-treatment, spiders were fed at libitum (i.e. every third day subadults received 5 flies, adult males 6 flies

and adult females 10 flies). This resulted in four experimental groups (2 populations x 2 treatments) for both males and females. In this species, earlier experiments showed adult females to have higher energetic requirements than males (F. Hendrickx, unpubl. data), hence the sex difference in food treatment. To ensure full adult development, spiders were tested not earlier than 3 days after final molt for males and 8 days for females. Females were tested at a later age as their maximal receptivity is achieved when they have acquired sufficient energy for egg development (Foelix 1996).

Body size of all spiders was measured as their maximum cephalothorax width (CTW), to the nearest 0.06 mm using a Wild M3 stereomicroscope with eyepiece graticule (Heerburg, Switserland). To account for measurement error, each measurement was repeated three times and the average value was used in subsequent analyses. After each mating trial, individuals were also weighed to the nearest 0.1mg (GalaxyTM 110 Ohaus; MASS). As adult mass does not remain constant during the adult phase (Hagstrum 1971), it was only used to verify the effect of stress at experimental group level and CTW was used as a covariate for individual size.

2.2 Mate choice experiment

To study effects of population of origin (R or P), stress treatment (C or S) and adult size (CTW) on mate choice, a single-male protocol (i.e. one male presented to a female) was applied as this best mimicked field conditions for *P. piraticus* (see also (Hendrickx *et al.* 2003) (Vanhook & Yates 1975; Nyffeler & Benz 1981;Larsen *et al.* 1994;Wilczek & Migula 1996;Hendrickx *et al.* 2004; Wilczek *et al.* 2008; Jung *et al.* 2008)). The test arena consisted of a transparent plastic box (11 x 11 cm, 5 cm high) with moistened graph paper lined at the bottom. Females were allowed to acclimatize for five minutes, after which a male was introduced at maximal distance from the female. If the male remained inactive for 10 minutes, it was replaced by another male from the same experimental group. If no copulations occurred within 15 minutes, the experiment was stopped since longer trials do not result in higher copulation rates in *P. piraticus* (D. Eraly, unpubl. data). If females remained unfertilized, they were tested

again the following day with another male from another experimental group, but not more than 5 times. After each trial, the graph paper was discarded and the box cleaned with ethanol to remove any chemical cues. A four-by-four fully factorial design with the factors 'stress treatment' and 'population of origin' (males and females) was adopted with random assignment of individuals to mating trials. In a first series of experiments (Mar-Apr 2006), effects of food stress on mate choice were compared between P and R spiders, resulting in 198 observations from 153 females (28 RC, 20 RS, 55 PC , 50 PS) and 92 males (22 RC, 16 RS, 27 PC, 27 PS). Because for population R the within population sample size was too small to test interaction effects, we decided to repeat the experiment with only spiders from population R in Mar-Apr 2007, resulting in 179 observations from 46 females (30 RC, 16 RS) and 49 males (22 RC, 27 RS). Trials in which males did not show courtship behavior were excluded from subsequent analyses. As some individuals died before measurements on CTW or Mass could be taken, sample sizes varied slightly among analyses.

2.3 Statistical analysis

We first verified whether the stress treatment had a negative effect on body size (both Mass and CTW) and compared this effect between populations with a two-way Anova (proc glm, SAS 9.1, SAS Institute Inc.©). The initial model contained all four-, three-, and two-way interactions. Non-significant interaction terms ($p>0.1$) were subsequently excluded in a stepwise procedure, starting with the highest order and least significant interactions.

During each mating trial, we observed whether or not copulation occurred, which comprised the most straightforward and relevant response variable for addressing our research questions. Because some experimental groups either contained a small number of observations or showed a uniform response (e.g. all RC females copulated), we relied on exact tests (LogXact and StatXact, Cytel Studio 8.0.0, Cytel Inc ©) to quantify the effects of stress and population of origin on copulation probability. To test for assortative mating (research question i) the effect of male and female

population origin, and particularly their interaction was of main interest, while for the effects of resource acquisition on mate choice (question ii) the effect of male and female stress treatment was tested. To study differential effects of stress on both populations (question iii), the interaction between population origin and stress treatment of both partners was most important. Since we used an exact test, all interactions of interest had to be tested separately with three-way interactions being the highest order possible.

To control for the effects of size (CTW) on the probability of copulation, this factor, as a continuous variable, together with stress treatment and population origin was analyzed using generalized linear mixed models (proc glimmix; SAS 9.1, SAS Institute Inc. ©). As the response variable is binomially distributed, a logit link function was used. Starting models contained all main factors and interactions. Again, model selection was done by removing non-significant interaction terms in a stepwise manner and starting with the highest interaction terms. As most males and some females were tested repeatedly, all analyses incorporated their identity as a random effect. The estimate variance components of this random factor were however not significantly larger than zero.

3 RESULTS

3.1 Phenotypic stress effects

To verify whether the applied stress treatment was effective, we analyzed phenotypic effects on cephalothorax width and body mass for spiders collected in 2006 and 2007. Female mass and CTW were significantly smaller under the food stress treatment compared to the control treatment, for spiders from both populations in both years (Figure 1 and Table 1). On average, female mass was 30% lower and female CTW 5.4% smaller for the stressed group. Male mass was 19% lower under food stress, whereas male CTW was not significantly affected by our treatment in either year or population (Figure 1 and Table 1). Significant differences in size between populations were only present for female mass (Table 1).

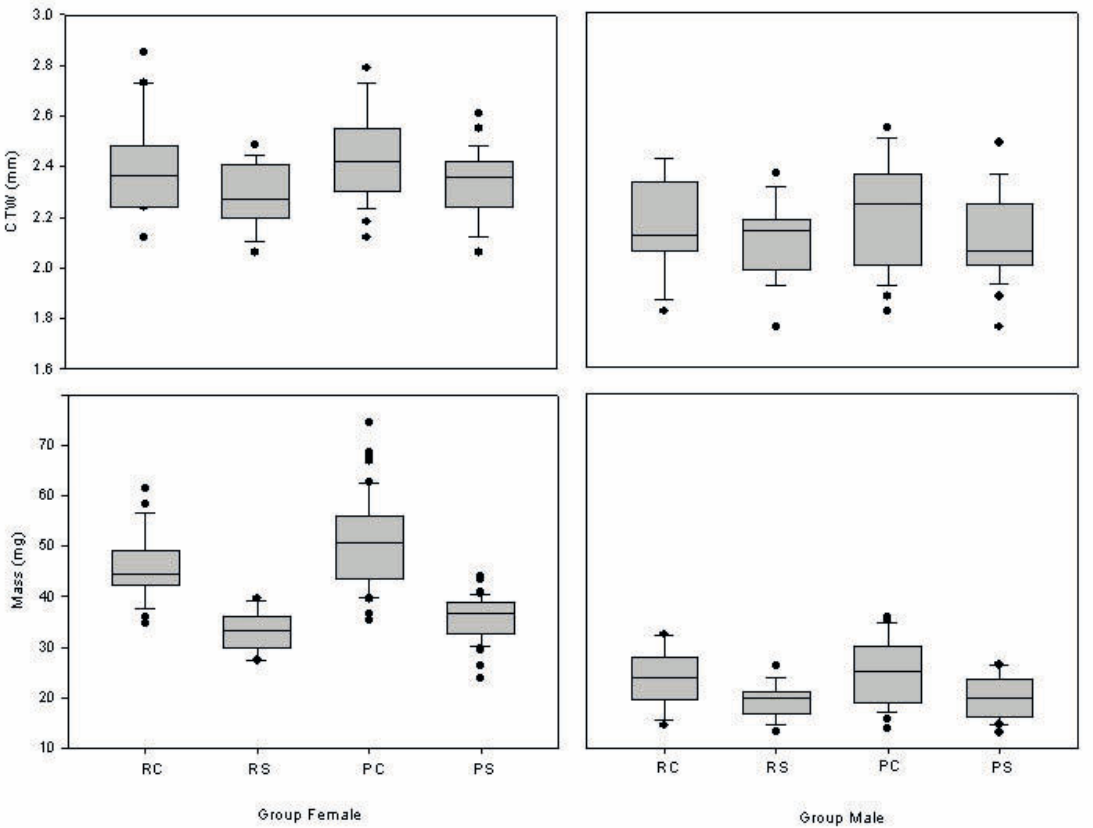

Figure 1 | Boxplot diagram of female and male cephalothorax width (CTW) and body mass (mass) for the four experimental groups. R=reference population, P= polluted population, C=control treatment, S= food stress treatment (data 2006). Graphs depict mean values (horizontal lines within boxes), upper and lower quartiles (top and bottom of boxes), 90th and 10 percentiles (vertical lines above and under the box) and outliers (dots).

(a)	**Mass**				**CTW**			
2006	$F_{1,140}$	P	N	Mean ± SE C or R/S or P	$F_{1,134}$	p	N	Mean ± SE C / S
stress	135.87	<0.0001	141	C 49.01±0.92 / S 35.04±0.51	20.11	<0.0001	135	C 2.43±0.02 / S 2.32±0.02
pop	11.37	0.001	141	R 40.52±1.87 / P 43.72±1.00	2.2	0.1	135	/
stress*pop	1.1	0.3	141	/	0.01	0.9	135	/
2007	$F_{1,45}$	P	N	Mean ± SE C / S	$F_{1,39}$	p	N	Mean ± SE C / S
stress	66.87	<0.0001	46	C 51.67±1.36 / S 35.35±0.96	10.43	0.003	40	C 2.53±0.03 / S 2.37±0.03
(b)	**Mass**				**CTW**			
2006	$F_{1,87}$	P	N	Mean ± SE C / S	$F_{1,93}$	p	N	Mean ± SE C / S
stress	18.21	<0.0001	88	24.93±0.85 / 20.01±0.53	3.41	0.07	94	/
pop	0.8	0.4	88	/	0.29	0.6	94	/
stress*pop	0.09	0.8	88	/	0.07	0.8	94	/
2007	$F_{1,44}$	P	N	Mean ± SE C / S	$F_{1,48}$	p	N	Mean ± SE C / S
stress	20.22	<0.0001	49	28.12±0.99/ 22.81±0.70	3.06	0.09	49	/

Table 1 | Effects of stress treatment (stress) and population of origin (pop) on female (a) and male (b) body mass (mass) and cephalothorax width (CTW) in both years. Means ± SE are given for both populations and stress treatments when differences where significant, first for the control treatment (C) or the reference population (R), hence for the stressed treatment (S) or the polluted population (P)

3.2 Stress and population effects on copulation probability

The probability of copulation did not significantly differ when both partners belonged to the same or different populations or treatments (Table 2; data 2006). Effects of food stress on the probability of copulation, however, differed between females from populations R and P (Table 2, and Figure 2). Food-deprived R females showed a significant reduction in copulation probability (100% to 60%). P females showed a lower copulation probability under the control treatment compared to R females but, in contrast to the latter, did not show a significant reduction in copulation probability under food stress (73 to 70%).

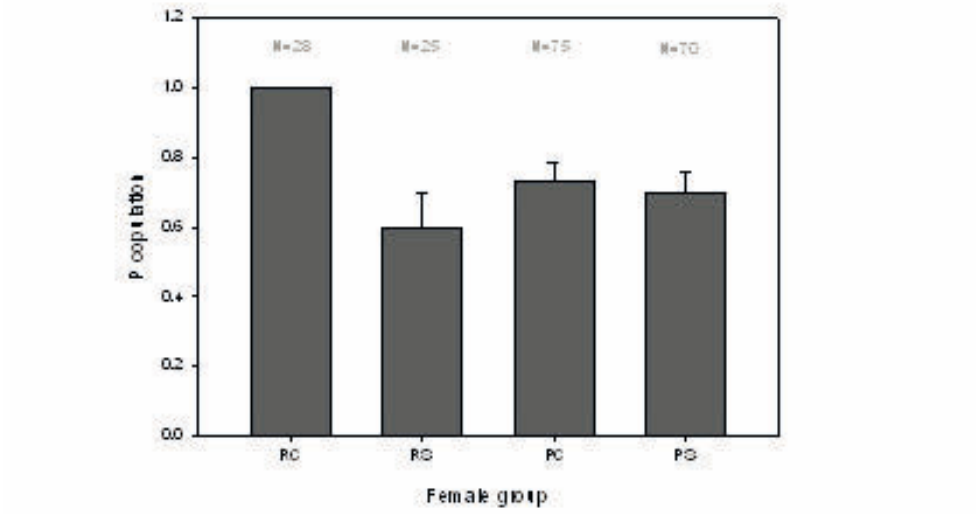

Figure 2 | Probability of copulation (mean ± SE) for the four experimental groups of females. R=reference population, P= polluted population, C=control treatment, S= food stress treatment (data 2006). For each group, the number of mating trials conducted is indicated by N.

Model term	P value
stressF	**0.0007**
popF	**0.002**
Stress	0.07
popM	1.0
popF*stressF	**0.001**
popF*popM	0.4
popF*stress	0.5
popM*stress	0.6
stressF*stress	1.0
stressF*popM	1.0

Table 2 | Exact p-values from the logistic regression model testing the effects of stress treatment (stress) and population of origin (pop) of both females (F) an males (M) on the probability of copulation (data 2006). Significant effects indicated in bold. N=198.

In the 2007 experiment, consisting only of trials with spiders of population R, the effect of female stress on the probability of copulation was also highly significant (from 95 to 40%; $F_{1,174}$=48.92, p<0.0001).

3.3 Body size effects on copulation probability

Because copulation probability was not directly affected by population of origin nor by male stress (see above), and to ensure sufficiently large sample sizes, these analyses only included the factors female and male CTW and female stress treatment and were conducted separately for R-R (only data of 2007, because for 2006 all RC females copulated) and P-P (data 2006) mating pairs.

For R mating pairs, the effect of male CTW on copulation probability differed significantly between female treatments (Table 3). We found the

probability of copulation to be independent of male CTW for the control females, but to decrease with increasing male CTW for food stressed females of all sizes (Figure 3).

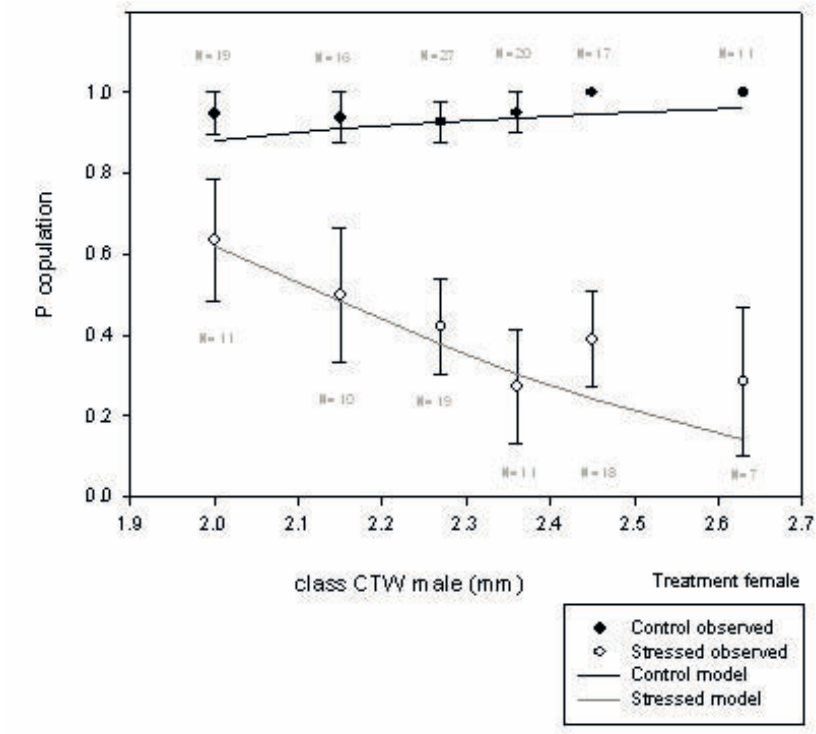

Figure 3 | Effect of male cephalothorax width (CTW) on the probability of copulation (mean ± SE) for R mating pairs with stressed and control females (data 2007). Male size was divided in classes, represented by the average of each class. Within each experimental group, the number of mating trials conducted is indicated by N. Lines depict model estimates, points depict observed values.

For P mating pairs, the effect of male CTW on copulation probability depended on female stress treatment as well as female CTW (Table 3, Figure 4). Under both female treatments, the largest males (average CTW 2.4 mm) were more likely to copulate with the largest females, and the smallest males (average CTW 1.8 mm) with the smallest females. For males of intermediate size, in contrast, the relation between male and female size and the probability of copulation depended on female treatment. Males with an average CTW of 2.2 mm had a higher probability of copulating with

small food-stressed females and with large control-treatment females. For the group of males with an average CTW of 2.0 mm the probability of copulation was higher with smaller females, but this relation was stronger with stressed females.

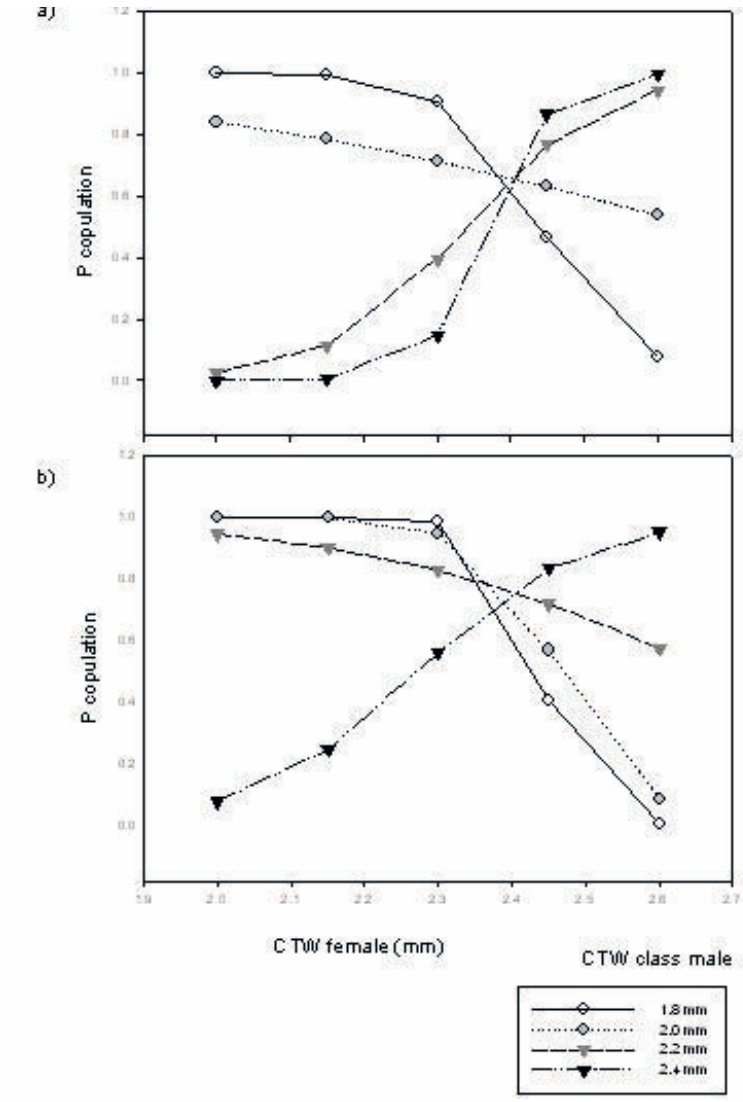

Figure 4 | Relationship between the probability of copulation and male and female cephalothorax width (CTW) for P mating pairs in (a) female control group, (b) female stressed group (data 2006). Male size was divided in classes, represented by the average of each class.

		R		P	
Parameter	DF	F	p	F	p
CTW male	**1**	**0.38**	**0.5**	**9.75**	**0.002**
Stress female	**1**	**2.06**	**0.1**	**4.22**	**0.04**
CTW male*stress female	1	3.69	0.03	0.01	0.9
CTW female	**1**	**0.17**	**0.7**	**9.3**	**0.002**
CTW male* CTW female	**1**	**0.57**	**0.5**	**9.51**	**0.002**
CTW female *stress female	**1**	**0.66**	**0.4**	**4.25**	**0.04**

Significant effects indicated in bold. N_R =177, N_P=59.

Table 3 | Results of a generalized linear model analysis on the effects of stress treatment (stress) and size of both partners on the probability of copulation within populations, conducted separately for population R (data 2007) and P (data 2006).

4 DISCUSSION

Our experiments could not show a preference of *P. piraticus* for spiders of their own population. Subjecting females to food stress significantly affected their mate choice, though the strength and direction of the response differed in relation to the population of origin. When females from an unpolluted reference population were food-deprived, they strongly reduced their probability of copulation and mated more frequently with smaller males. This trend was not evident for females from the polluted population, where the probability of copulation was lower than for reference females when food was abundant, but remained unaltered when females were exposed to food stress. Individuals from the polluted population, but not from the reference population, showed size-assortative mating, most strongly so when females were food-deprived.

Food stress has earlier been shown to adversely affect both male and female condition, courtship behavior, and attractiveness to individuals of

the opposite sex (Andersson 1994). In our study, phenotypic effects of food stress were stronger in females than in males, and food-stressing the latter did not significantly affect the probability of copulation. In *P. piraticus*, females have higher energetic needs than males and experience energetic constraints to mating earlier, i.e. under lower levels of food deprivation (F. Hendrickx, unpubl. data). Although we exposed males to a more severe stress treatment, the smaller treatment effects on male phenotype and mating probability suggest that males experienced less stress than females.

When females from the reference population were food-stressed, their responsiveness to copulate decreased (and age of first copulation delayed; D. Eraly, unpubl. data), providing experimental evidence for the passive initiation hypothesis (Crespi, 1989; Hingle et al. 2001; Hebets et al. 2008). This hypothesis states that mate choice does not result from active choice but rather from external factors affecting mating propensity, in this case the available energy for egg production. As shown by Hendrickx et al. (2003), female *P. piraticus* that live under more favorable conditions show a higher fecundity. Under such benign conditions, female fecundity is mainly constrained by body size, and females can be expected to postpone mating until sufficient resources have been obtained for egg development (in spiders, egg fertilization generally takes place when eggs are fully developed (Foelix 1996; Uetz and Norton 2007)). Females in polluted sites, in contrast, are known to produce smaller egg cocoons relative to their weight (Hendrickx et al. 2003) and these females apparently do not wait to initiate fertilization until a maximal body mass is obtained. Such shift in life-history strategy, which is considered adaptive (Hendrickx et al. 2003), may explain the apparent lack of an experimental food deprivation effect in responsiveness for females from the polluted population.

In addition to a reduction in responsiveness to copulate, stressed females from the reference population showed a higher preference for smaller mates. This finding applies at group level since our experiment was not designed to study individual preferences (Wagner 1998). In absence of stress, a larger body size is generally considered to be advantageous, i.e. to correlate positively with reproductive success (Honek 1993; Olsson 1993;

Blackenhorn 2000; Danielson-Francois et al. 2002; Hendrickx and Maelfait 2003) and with developmental condition (e.g. Hebets et al 2008). Stressful conditions, however, may shift the cost-benefit balance towards smaller sizes, as the latter require lower energy budgets and shorter growth intervals, provide higher agility and enhanced mate-searching ability, and incur lower costs of reproduction and predator visibility (Blanckenhorn 2000). Field caught *P. piraticus* from polluted habitats indeed are on average smaller (CTW and mass) than those from more pristine habitats (Hendrickx et al. 2003). Effects of size on mate choice may also be explained by differences in vigor during male courtship. Moreover, relationships between size and mating success are likely to depend on energy balance, and hence, resource availability (Kotiaho 2000; Uetz et al. 2002; Blanckenhorn et al. 2008). Since relationships between courtship vigor and size have not yet been studied in *P. piraticus*, the relative importance of this mechanism for mate choice remains to be quantified. Alternatively, female preference for smaller males under food stress may reflect an increased response to moving objects when females are hungry. A study of Mayntz and Toft (2006) on a wolf spider showed that spiders attacked conspecifics more when starved for a longer period. As suggested by West-Eberhard (2003), female preference and male courtship traits are likely to be evolved as duplicated characters of prey detection and movement behavior. At first, a male may attract attention as a potential prey and thereby increase its probability of copulation. Smaller males more likely resemble prey and this may additionally explain the greater tendency of hungry females to approach them. In accordance, all but the largest stressed females of population R also preferentially mated with smaller males. For mating pairs of population P, size-assortative mating was most pronounced when partners differed most strongly in size. This would also imply that the strongest discrimination in mate choice would occur between the most divergent populations (in size). Size-assortative mating is known to be a general mating pattern (Crespi 1989, Rowe and Arnqvist 1996; Taborsky et al 2009) and it has been observed in both vertebrates and invertebrates: various taxa of spiders (e.g. Masumoto 1999; Hoefler 2007), water striders (e.g. Rowe and Arnqvist 2002; Ortigosa and Rowe 2002), beetles (e.g. Harari et al. 1999), snails (e.g. Cruz et al. 2004), fish (e.g. Schluter and Nagel 1995 and Taborsky et al. 2009), lizards (e.g.

Olsson 1993) and birds (e.g. Helfenstein et al 2004). Our results confirm the importance of body size in the mating pattern of *P. piraticus*. The absence of significant size differences between both populations for males and females of our laboratory-reared spiders can also be a consistent explanation for the lack of population differentiation in mating preference.

While a lower responsiveness for food-stressed females was shown in a variety of taxa, such as water striders (Rowe et al. 1994; Ortigosa and Rowe 2002), field crickets (Hunt et al. 2005), cockroaches (Clark et al. 1997), stalk-eyed flies (Hingle et al. 2001), and guppies (Syriatowicz and Brooks 2004), other studies either failed to show a significant relationship (Archard et al. 2006; Tigreros and Switzer 2008) or found an opposite trend (Cratsley and Lewis 2003). In the latter study on fireflies, males provide females with a protein-rich spermatophore, a behavior which explains the opposite results. This illustrates the importance of taking the mating system of the study species into account when explaining the results (Bonduriansky 2001). Recent studies on spiders also showed highly diverse effects of female condition on mate choice. For instance, contrary to theoretical predictions and our results, Hebets et al. (2008) found that for *Schizocosa sp.* their diet treatment could not be shown to affect female responsiveness, and this was also the case in a study on another wolf spider *Pardosa milvina* (Wilder and Rypstra 2008). However, female *Schizocosa sp.* that were raised on a high-quality diet showed a higher preference for males from the same treatment, while females raised on a low-quality diet showed no preference, a finding opposite to ours. Apart from the fact that these studies manipulated both food quantity and quality, *Pardosa* and *Schizocosa* males show elaborate courting behavior and *Schizocosa* males have conspicuous brushes on their legs which are considered secondary sexual traits (Uetz and Roberts 2002), both of which are absent in males of the species in our study. These male traits are more condition-dependent and as a consequence provide more reliable cues for female preference (Uetz and Roberts 2002).

In conclusion, results from our study support the prediction that variation in body condition, driven by local ecological factors, may affect mating rates, and hence, sexual selection (Ortigosa and Rowe 2002; Vines and

Schluter 2006; Hendry et al. 2008). As individuals from a single gene pool produced different phenotypes (body sizes in this case) when subjected to food stress, this also provides evidence for phenotypic plasticity and its role in establishing reproductive isolation when two populations are subjected to different environments that induce this plasticity (West-Eberhard 2005; Crispo 2008). However, as we only studied two populations at the extremes of a pollution gradient, studying more intermediates and replicates in the future is necessary to confirm our findings. While results from this study fail to show differences in mate choice in relation to population of origin *per se*, preference of smaller mates by food-stressed females may ultimately reinforce population differentiation.

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Author's contribution

Debbie Eraly and Frederik Hendrickx conceived and designed the study and wrote the manuscript together with Luc Lens. Debbie Eraly maintained the spiders in the lab, executed all mating trials and did the measurements, with the help of some students as mentioned above. Debbie Eraly and Frederik Hendrickx analyzed the data. Thierry Backeljau provided usefull feedback on the manuscript.

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GENERAL DISCUSSION

1 INTEGRATION OF RESULTS

1.1 Overview of the key results for *Pardosa saltans*

We summarized the most important results of this study for *Pardosa saltans* in Table 1 and discuss them point by point in the following paragraphs. Afterwards we integrate and discuss the most obvious patterns.

	Metal body burden		**Physiological defense**			
			field		laboratory	
Population mean	Cd	Zn	MTLP	free metals	MTLP +Cd	MTLP - Cd
CH-R1	3.98	85.57	6.79	0.26	12.76	7.47
CH-R2	3.76	107.59	6.33	0.28	12.66	7.4
CM-R	1.80	100.53	7.04	0.32	10.82	8.19
CH-P1	20.91	152.61	8.14	0.40	12.13	6.65
CH-P2	8.71	158.43	6.63	0.39	13.66	9.22
CM-P	3.29	133.73	6.16	0.28	11.76	7.57
Population differences and pairwise significant (p<0.005)	**p<0.0001** CH-P1, CH-P2> CM-P, CH-R1,CH-R2 > CM-R	**p=0.0016** P>CH-R1	p=0.27	**p=0.011** CH-P1, CH-P2>CH-R1	MTLP conc: p=0.66 MTLP increase upon exposure: p=0.76	
Correlation with Cd	/	**p=0.0024 pos**	p=0.33	/	p=0.94	p=0.48

Table 1 | Overview of the key results of this research: mean, p-value for ANOVA between populations and and p-value for correlation with Cd concentrations.

	Life history traits field populations					
Population mean	Mass male	Mass female	Mass cocoon	Reprod. allocation	Fecun-dity	Mass egg
CH-R1	15.03	26.88	20.67	0.78	41.40	0.52
CH-R2	12.51	22.84	16.21	0.73	34.71	0.49
CM-R	/	/	/	/	/	/
CH-P1	11.87	18.73	14.04	0.75	27.29	0.52
CH-P2	11.45	22.83	20.65	0.93	39.72	0.53
CM-P	/	/	/	/	/	/
Population differences -pairwise significant (p<0.005)	**p<0.0001** CH-R1> CH-R2, CH-P1, CH-P2	**p<0.0001** CH-R1> CH-R2, CHP2> CH-P1	**p<0.0001** CH-R1, CH-P2> CH-R2> CH-P1	**p<0.0001** CH-P2> CH-R1, CH-R2, CH-P1	**p<0.0001** CH-R1, CH-P2> CH-R2> CH-P1	**p=0.029** CH-P2< CH-R2
Correlation with Cd	**p=0.05 neg**	**p=0.01 neg**	p=0.1	p=0.1	p=0.1	**p=0.015 pos**

	Genetics		Mate choice
Population mean	Polymorphic Loci %	Expected Heterozygosity (Hj)	Popuation origin of preferred partner for female
CH-R1	91.7	0.34	CH-R1 > CH-P1
CH-R2	94.8	0.40	CH-R2 = CH-P2
CM-R	95.8	0.37	
CH-P1	91.1	0.34	CH-P1 = CH-R1
CH-P2	83.9	0.35	CH-P2 = CH-R2
CM-P	97.4	0.38	
Population differences (p<0.005)	Hj: CH-R2 highest; CH-P1 lowest PhiPT: all pairwise different except CM-P and CH-R2, biggest between CH-R1/CM-R and CH-P1/CH-P2		**p=0.014 (I_{psi})**
Correlation with Cd	p=0.39	p=0.23	/

1.2 Metal exposure

Since differences in metal concentration in field study populations are a prerequisite for the objectives of this research, we first compared metal concentrations in field populations of *Pardosa saltans* (chapter 1). The study populations are inhabiting sites exposed to metals by historical mining activities. Putative exposed and reference sites were selected based on the presence and absence of metalliferous flora respectively and three pairs of both a polluted and reference site were selected to compose a replicated design. In line with the expectations, the metal concentrations found in the exposed populations were markedly elevated compared to the reference populations, especially for Cd. For Cu the trends were reversed and highest in CH-R1 and lowest in the polluted populations. While Zn and Cu are considered essential for spiders (Jung & Lee 2012), Cd is regarded as a non-essential element to which the affinity of Metallothionein Like Proteins (MTLPs) is highest. Together with the finding that in our study Cd concentrations were elevated with a factor 10, we considered Cd to be the main stressor in this species. However, to demonstrate the biological significance of the exposure to measured Cd, Zn and Cu concentrations an ecotoxicological study determining lethal concentrations (LC 50) would be needed. The observed Cd body burdens are of the same magnitude to those found in other wolf spiders inhabiting severely polluted sites (Rabitsch 1995; Wilczek & Babczynska 2000; Heikens *et al.* 2001; Hendrickx *et al.* 2003; Wilczek *et al.* 2004; Wilczek *et al.* 2008) and even higher (Wilczek & Migula 1996; Wilczek *et al.* 2004; Jung *et al.* 2007; Jung & Lee 2012) taken into account a wet weight / dry weight ratio of 4. However, these concentrations are still well below reported acute lethal concentrations in another study on a Lycosid spider (Jung *et al.* 2007). With exception of the

1.3 Life history

The assessment for effects of metal exposure on key life history traits in the field populations (chapter 1) showed males to be smaller (cephalothorax width), lighter and under poorer condition when average Cd body burden of the populations was higher. Females from populations with a higher internal Cd concentration had a reduced mass, but for

cephalothorax width and condition only the most polluted populations differed from those of CH-R1 females. However, for most reproductive parameters the results were not straightforward and did not correlate with Cd burden. Cocoon mass was lowest for the most polluted population CH-P1, but in the population CH-P2 it was as high as in CH-R1, while in CH-R2 it was lower than in these two populations. Reproductive allocation was highest in CH-R1, but also in CH-P2. Fecundity neither correlated with Cd concentration. However, egg mass did correlate positively with Cd concentration. In conclusion, only for the most contaminated population CH-P1, life history alterations were as expected from life history theory (Stearns 1992; Roff 1992) and similar to those found by Hendrickx *et al.* (2003), but rather ambiguous for the remaining populations.

Life history changes due to metal pollution have been showed in different invertebrate taxa and different theories for their adaptive value have been formulated (Posthuma & Van Straalen 1993). Life history theory indeed predicts that a lower energy availability would lead to slower growth and lower reproductive output. However, this is expected to be partially compensated by producing larger progeny or eggs if individual fitness scales nonlinear with progeny size (Smith & Fretwell 1974; Tamate & Maekawa 2000). In accordance with life history theory females of the most polluted population in our study did produce the largest eggs and their reproductive output was clearly lower. Result of this field study indicates potential adaptive responses to metals, but probably only when a certain threshold is reached.

However, results from this study do not allow to make inferences on putative genetic changes that occurred in these populations in relation to metal exposure. Besides local adaptation, the patterns can also be due to a plastic response related to energy availability in the field. To investigate the respective roles of a pure plastic response versus genetic adaptation and their interaction, a common garden experiment was conducted with spider young raised in the laboratory descending from mothers collected in the same populations as the field study (chapter 2). When exposing spiderlings from 2 polluted and 2 reference populations to Cd spiked flies, internal Cd concentration increased similarly in both groups. The Cd

concentrations of the unexposed groups were remarkably lower than for the reference field populations (on average 0.42 versus 3.20 µg/g ww respectively). Remarkably, we also observed a decrease in Zn and Cu concentration under Cd exposure, indicating that exposure to a specific metal also alters general metabolic functions. In terrestrial invertebrates, Cu and Zn can be regulated to a certain degree and Cu is essential in hemocyanin (Foelix, 1996; Heikens *et al.* 2001; Babczynska *et al.* 2011), However, since Cd concentration increased almost tenfold, the uptake reduction was not sufficient to keep Cd concentrations within normal limits. Despite these elevated Cd concentrations, growth an survival appeared unaffected by the treatment. This is remarkable since Cd concentrations in the laboratory exposed individuals was almost three times higher (average 59.39 µg/g ww) than observed in the most contaminated field population (average 20.91 µg/g ww). Growth and survival in the laboratory also did not differ between exposed and unexposed populations. Potential costs of tolerance or trade-offs and adaptation to metals in the field exposed populations could thus not be uncovered from this laboratory study.

1.4 Metallothioneins

To assess if metal exposure in the field and in the laboratory indeed induces the expression of physiological defense mechanism, we further examined the concentration of Metallothionein Like Proteins (MTLPs) (Mason & Jenkins 1996; Park *et al.* 2001; Kohler 2002; Santiago-Rivas *et al.* 2007; Babczynska *et al.* 2011). Results of the field survey (chapter 1) showed that MTLP concentrations did not differ between polluted and reference populations, suggesting that MTLPs do not show a higher constitutive expression in polluted areas. This implies a larger concentration of free metals in CH-P1 and CH-P2 than in the other populations when assuming that MTLP's are the only mechanism to sequester metals. Spiders are known to have other metal defense mechanisms like storage in insoluble form (granules), repartitioning to the hepatopancreas, glutathione and esterase pathways. However these often involve MTLPs or interact with the MTLP metabolism making it hard to differentiate the different mechanisms (Posthuma & Van Straalen 1993;

Viarengo & Nott 1993; Mason & Jenkins 1996; Hensbergen *et al.* 2000; Wilczek & Babczynska 2000; Desouky 2006; Morgan *et al.* 2007; Wilczek *et al.* 2008). It thus still remains to be studied which other defense mechanisms are at play in *P. saltans*, and to what degree they protect the spiders from detrimental effects of metals. The presence of intracellular granulas storing metals could be quantified by X-ray microanalysis or infrared spetrocopy analysis (as in Babczyska *et al.* 2011) and could be combined with MLTLP measurements under different exposure degrees to see to what degree they correlate.

As shown in other studies on spiders, and in contrast with insects, the surplus of metals are not excreted by spiders (Hendrickx *et al.* 2003). In conclusion, increased MTLP production in naturally exposed population could not be demonstrated.

However, when exposing *Pardosa saltans* to Cd under controlled laboratory conditions (chapter 2), MTLP concentrations significantly increased up to 12.30 nmol MTLP/ g ww (compared to 7.75 in untreated individuals) under high Cd stress. As this increase in MTLP production did not differ between individuals originating from exposed and reference populations (chapter 2), spiders from metal polluted populations do not show a higher constituve MTLP production, nor a stronger inducability. This also suggests that the field concentrations of Cd were likely too low to elicit this response. MTLP concentrations in the control treatment were comparable to field concentrations (6.83 ± 0.25 and 7.75 ± 041 nmol MT/ g ww, respectively). MTLPs are known to be also induced by stressors other than metals and perform several functions with regard to the general metabolism (Stegeman *et al.* 1992; Roesijadi 1996; Amiard *et al.* 2006). Therefor they are often considered unsuitable as specific biomarkers for heavy metal pollution (Mouneyrac *et al.* 2002; Santiago-Rivas *et al.* 2007).

Taken all together MTLP production not only appeared a highly plastic response in this species, but apparently also did not affect resources available since the studied life history parameters were unaffected by the Cd treatment. An exposure study on metal polluted populations of *Orchesella cincta* also showed no effects on survival though MTLP expression levels were raised (Timmermans *et al.* 2005). A longer term

exposure experiment on this species would provide more insights on long term effects of metals, though a common garden experiment in the fieldwould even be more interesting since it also takes synergistic effects into account. However this would be logistical challengen taken into account our experience with earlier field experiments (see §2.1).

Our conclusion, however, can not be extrapolated directly to field conditions as spiders were fed ad libitum and this extra intake of energy could compensate for energy spend on metal defense. These results further question the use of MTLPs as a reliable biomarker to assess detrimental effects of metal stress in natural populations (Roesijadi 1996; Mouneyrac *et al.* 2002; Amiard *et al.* 2006; Forbes *et al.* 2006; Morgan *et al.* 2007; Santiago-Rivas *et al.* 2007). The fact that the most polluted field populations did show changes in some important life history traits, indicates that they do are affected by the metals in their environment, though their MTLP level did not differ.

1.5 Genetic patterns

To study the patterns of genetic diversity and differentiation between differentially exposed populations, we performed a genome wide screening study using AFLP markers. This study provided some important new insights, albeit still very superficially since anonymous and dominant AFLP markers were used (chapter 3).

In general populations exposed to pollutants are predicted to experience a loss of genetic diversity, especially through strong selection against sensitive genotypes, that decreases effective population size and increases genetic drift. However, evidence against this theory is accumulating (Van Straalen & Timmermans 2002; Dibattista 2008; Ribeiro *et al.* 2012). No evidence for genetic erosion due to metal pollution was present in the studied populations since neither of the population genetic diversity measures correlated with metal concentration. Though, as was evident from the other parameters studied, the most heavily polluted population CH-P1 once again showed the lowest expected genetic diversity in terms of expected heterozygosity. However, contrary to life history parameters, CH-R1 did not differ from CH-P1 in genetic diversity. This can be explained by

the fact that *P. saltans* colonizes forest clearings, which are generally of a transient nature. The high metal concentrations of the polluted sites in contrast do not permit forest regrowth and therefore persist for several decades. Hence, populations from polluted sites in general have a much longer history allowing a longer influx of migrant individuals and consequently, a higher genetic diversity that counteracts potential diversity reducing effects as a result from strong selection. Thus, the reference sites can be considered to consist of ephemeral populations, expected to be founded by only a few individuals, and thus to be subjected more strongly to accidental founder events and thus reducing genetic diversity. Such counterintuitive results with regard to effects of metal exposure on genetic diversity were also reported in reviews of Van Straalen & Timmermans (2002) and Williams & Oleksiak (2008) where six out of 17 studies also found no reduction in genetic diversity in populations exposed to different kinds of toxicants using different genetic markers. In a quantitative genetic study on *P. piraticus,* Hendrickx *et al.* (2008) showed that cadmium contamination strongly decreased the heritability for growth, but only for the reference population. For the contaminated populations, heritabilities for this life history trait were low, and not affected by the applied cadmium treatment.

Genetic differentiation clearly was largest between polluted and reference populations. This pattern accords with observations in life history differentiation. Since populations from different geographic clusters were used and metal contamination was not linked to this spatial structuring, gene flow appears to be partially restricted. Mechanisms that can result in such a pattern are (i) very strong selection against maladapted individuals, or (ii) repeated selection of identical alleles in exposed populations resulting in a strong differentiation at a few loci among differentially exposed populations (i.e. outlier loci) (Hohenlohe *et al.* 2010). Different outlier loci were detected with two different methods in multiple population comparisons, including populations from the two geographic clusters and in independent comparisons excluding within group comparisons. The occurrence of outlier loci linked to metal pollution could also explain the absence of a pattern of genetic erosion since the divergence at a few loci only suggests that the influx of alleles into

polluted populations is still possible. The latter is supported by the mate choice experiments which showed no evidence of absolute selection against mates from other populations.

The results from these analyses thus suggest that exposure to metal pollution in this metapopulation selects identical alleles in the face of homogenizing gene flow thereby maintaining genetic variation. While from the Cd-exposure study we would conclude no local adaptation to be present since naturally exposed populations did not show any difference in survival and growth between the spiders originating from polluted compared to reference populations. In contrast, the population genetic study does show the presence of genetic differentiation and outlier loci to be present. This could imply that genetic studies might be better suited to detect (early) signs of metal stress on natural populations. Another potential explanation for this discrepancy are the choice of traits measured in the laboratory study i.e. growth and survival. Moreover the power to detect differences might have been low on this dataset that only included a few populations. The fact that MTLP production increased with increasing Cd exposure, but growth did not, somehow illustrates that physiological responses to cope with metals are hardly reflected in life history traits. Both seem plausible since the variability between individuals of the same treatment or population was very high. To conclude, the most plausible explanation for this discrepancy is that the benign laboratory environment does not realistically mimics the natural situation, where more stressor and indirect effects of metals can be at play.

1.6 Mate choice

The patterns of genetic differentiation and occurrence of outlier loci suggest that some reproductive barriers exist between the exposed and reference populations. We explored this hypothesis more directly by exposing spiders from different populations from a metal exposure gradient to mate choice experiments in the laboratory. This aspect of local adaptation and sexual selection is largely neglected in most of the research on environmental stress (Bonduriansky 2011).

A first and most straightforward mechanism explaining how adaptation to different environments could induce a reproductive barrier is a shift in reproductive timing or heterochrony (Winterer & Weis 2004). Populations reaching sexual maturity at different times could already experience an important barrier to intrapopulation matings, even in the presence of migration events. Effects of stress on phenology or reproductive timing have been studied in plants (Griffith & Watson 2005; Vekemans & Lefebvre 1997), but to a lesser extent in animals, with the exception of temperature (stress). Based on our studies on phenology in *P. saltans*, it is unlikely that heterochrony further drives population divergence as the timing at which females and males became adult did not correlate with metal burden (Chapter 1). Yet, in line with the results of the other life history traits, mature females of the most severely exposed population tended to maturate later compared to those of reference population CH-R1. However more replicas (populations and years) should be included to confirm this trend.

Another important driver of reproductive isolation is divergence in female mate choice (Schneider & Lubin 1998; Andersson & Simmons 2006; Albo *et al.* 2011). Since for *Pirata piraticus* differences in life history traits due to metal pollution are well studied (Hendrickx *et al.* 2008), we primarily made use of this study system to address this relatively unexplored field of research (chapter 4). Two populations located at both extremes of the pollution and life history trait gradient (Hendrickx *et al.* 2003) were differentially exposed to stress. We opted to use food stress rather than metal stress as it is much more severe, making it more straightforward to assess the relatively unexplored effect of stress on mate choice. We choose not to apply a choice experiment, since this best mimics the natural situation, although it does not allow to compare individual female choice between different potential mates at the same time.

Besides this experiment with *P. piraticus,* we also performed some preliminary analysis (and therefore not included as a separate chapter) on mate choice in *Pardosa saltans*. Spiders were collected in four populations, 2 polluted and 2 reference populations, also sampled for the research described in chapter 1 and 2, and females were presented 2 males of

different populations. We summarize the most important results of this study in table 2.

			pop M				
			CH-P1	CH-R1	CH-P2	CH-R2	average mating
		N	77	77	72	72	propensity
Pop F	CH-P1	36	15	12			0.75
	CH-R1	41	7	22			0.71
	CH-P2	45			16	18	0.76
	CH-R2	27			8	8	0.59
total		149					0.70

Table 2 | Mating frequencies (number of observed copulations) for the different combinations of females (rows) and males (columns) with total numbers of males and females tested respectively in the first row and colum.

Results show a higher mating propensity in *P. piraticus*, however different between populations (under control treatment; 100% R and 73% P) than for *P. saltans* (71%) confirming the expectation that *P. saltans* females are more selective. No preference for mates of her own population in *P. piraticus* was found, while this does was the case for the most strongly divergent populations in *P. saltans* (Table 1; combination CH-R1 x CH-P1, index for sexual isolation I_{psi}=0.33, p=0.014; D. Eraly, unpublished data*)*. In contrast to *P. saltans*, *P. piraticus* female populations did differ in mating propensity, regardless of male population, which was higher for reference females, but was severely lowered when exposed to stress in this group. For females of the polluted population this decline was not observed. This could be interpreted as females of contaminated populations to be more stress resistant. The reduced responsiveness in stressed females from the pristine population could be explained by a slower maturation of the eggs, a priority to foraging or a reduced motivation to mate when experiencing a reduced condition, especially since in this species females tend to mate only once. The effects of (food) stress on mate choice between stress-adapted and non-adapted populations in *P. piraticus*

differed. Only the females of the reference population showed a clear reduction in mating propensity when food deprived, while in the contaminated population, responsiveness was overall lower. Condition-dependence of female mate choice thus seems to be at play in this species, and shows that mating is more costly when individuals are stressed and increases selectivity. A lower responsiveness for food-stressed females was also apparent in other studies on water striders (Rowe *et al.* 1994; Ortigosa & Rowe 2002), field crickets (Hunt *et al.* 2005), guppies (Syriatowicz & Brooks 2004), cockroaches (Clark *et al.* 1997), stalk-eyed flies (Hingle *et al.* 2001), but not in other studies on guppies (Archard *et al.* 2006), Japanese beetles (Tigreros & Switzer 2008) and fireflies (Cratsley & Lewis 2003).

Though both species belong to the same spider family *Lycosidae* and are exposed to a comparable stressor in the field (metals), some important differences need to be pinpointed. First of all, *P. saltans* males show a very pronounced courtship display, which contrasts strongly with the courtship behavior of *P. piraticus* where it is very subtle or even absent. Therefore this behavior in *P. saltans* can also can be a more important cue for mate choice. This already indicates that studying this aspect of *P. saltans* biology would be very informative (see future prospects). Another important difference is the duration of the copulation that in *P. saltans* lasts for several hours (268.84 ± 4.14 minutes) while for *P. piraticus* it only lasted for a few seconds (9,31 ± 0.57 sec, D. Eraly, unpublished) allowing a lot more opportunity for cryptic female mate choice in *P. saltans*. Moreover the impact of male size should be larger in *P. saltans* since the female is carrying the male for several hours, increasing carrying costs (Sih *et al.* 2002) and increasing visibility to predators. The fact that in the *P. saltans* study replicate populations were studied makes its conclusions more sound and it also shows that very different conclusions would have been drawn when only one of the combinations had been studied. In future studies on *P. piraticus* more populations should be included, though now the most diverged populations were analyzed and therefore the strongest effects would be expected but both populations also differ in other environmental parameters than metal burden.

Indications for size-assortative mating were not straightforward. All but the largest stressed females of population R preferentially mated with smaller males. For mating pairs of population P, size-assortative mating was most pronounced when partners differed most strongly in size. This would also imply that the strongest discrimination in mate choice would occur between the most divergent populations (in size). Stressed females from the reference population showed a higher preference for smaller mates. Since field caught *P. piraticus* from polluted habitats indeed are on average smaller habitats (Hendrickx et al. 2003) this could provide an indirect mechanism for population assortative mating.

The results confirm that environmental changes that influence resource availability, and thus plasticity in related traits, can influence mating behavior and thus have the potential to affect sexual isolation and result in population differentiation (Ortigosa & Rowe 2002; Vines & Schluter 2006; Hendry *et al.* 2008). Under natural conditions, the effects of stress can even be expected to be more pronounced since the tested spiders were subjected to only a single stressor in the final stages of their lives. Under natural circumstances, they are most likely to disperse as young juveniles. (Nyffeler & Benz 1981). Other studies concerning assortative mating experiments in the laboratory give mixed results (Mooers *et al.* 1999). Field studies on divergent ecological selection in *Drosophila* support the hypothesis of assortative mate choice as a by-product of adaptation to a heterogenous environment (Korol *et al.* 2000). But to fully settle the ecological speciation hypothesis it would be necessary to do tests more populations subjected to comparable environmental circumstances.

1.7 Integration of the main results

To conclude, I here attempt to integrate the main results of the different subsections which were not always in support of each other.

First, adverse effects of metals appear to be only expressed if they exceed a particular threshold. This is concluded from the following observations: (i) for *P. saltans* clear changes in life history traits were observed only for the

most polluted population; (ii) MTLP production is only increased under high Cd exposure, but not at intermediate levels measured in the field populations (chapter 2) and (iii) genetic diversity was lowest in the most polluted population.

Secondly, in the natural habitat, effects of metals might be more pronounced compared to in the laboratory, where no measurable effects on growth or survival were apparent This may have several causes: (i) importance of indirect effects of metals in the field, mostly through resource availability; (ii) synergetic effects of different stressors in the field; (iii) absence of natural patterns in the artificial laboratory. The effects of metal pollution could be rather indirect i.e. that reduced resource availability in field exposed populations also emerges from reduced energy availability through reduced prey availability, decreased energetic value of the prey and changes in species composition (Tranvik *et al.* 1993; Spurgeon *et al.* 1996; Klerks 2002; Jung *et al.* 2008; Clements & Rohr 2009). Species composition of invertebrates was indeed shown to change under metal stress in different studies (Bengtsson & Rundgren 1984; Read *et al.* 1998; Lock *et al.* 2003; Creamer *et al.* 2008). Since female mass affects reproductive output, effects on energy availability can have a profound impact on population growth. Moreover different toxicants acting together can cause synergetic effects on natural populations (Jones & Hopkin 1998; Clements & Rohr 2009) that are difficult to mimic under laboratory circumstances and thus stress the importance of field tests (Lock *et al.* 2003; Amiard *et al.* 2006; Clements & Rohr 2009). Therefore the effects of metals are probably only poorly mimicked in the laboratory by just feeding Cd spiked flies. The artificial circumstances in the laboratory could also obscure subtle differences in growth between the populations. In the Cd exposure study on P. *saltans* for example mass of males at age 35 and 50 days and of females at 50 days did not differ between populations (taken into account differences in treatment). Only female mass at age 35 days was shown to differ between the populations with CH-R1 and CH-P2 females weighing more than CH-P1 and CM-P females (D. Eraly, unpublished data). Different spider species, and also *P. saltans*, are known to occur in different cohorts and some juveniles go into diapause before maturing as others mature in the same year (F. Hendrickx, personal communication). If

the external trigger of diapause is lacking under laboratory conditions, growth patterns can be strongly disrupted, which seriously affect growth patterns and other related traits.

Phenotypic plasticity is another important mechanism that is assumed to explain the patterns found. It allows individuals to cope with different and variable ecological factors. Especially in a patchy environment where animals are likely to face different values of ecological parameters, plasticity is an advantage. Overall costs to plasticity appear to be rather low (Dewitt *et al.* 1998; Hoffmann & Hewa-Kapuge 2000; Sultan & Spencer 2002; Kristensen *et al.* 2008; Terblanche & Kleynhans 2009; Van Buskirk & Steiner 2009), especially in a metapopulation context, as this study (chapter 2) also confirms. We could not find evidence for phenotypical plasticity to be geneticaly adapted since both naturaly exposed and reference populations showed a comparable response to metal exposure.

1.8 Conclusion

Considering all the results of these studies together, evidence for local adaptation to metal exposure seems rather weak in *Pardosa saltans*. Though the most convincing measures, genetic differentiation and presence of outlier loci, support a pattern of divergence linked to metal exposure, this is not the case for common garden experiments, MTLP concentrations and life history traits considering the whole gradient. When only the extreme populations are compared however, the patterns support theory on life history adaptations under stress.

Evidence for local adaptation in *Pirata piraticus* that was already established earlier (Hendrickx *et al.* 2008), was not confirmed by the mate choice study we conducted, though indirectly, through the process of size-assortative mating, population differentiation could be reinforced

Our study species thus seemed moderately impacted by metals, however, without threatening its long-term survival. The increased differentiation in this species due to metal exposure could be interpreted as a increase in (genetic) biodiversity. However, we do not intent to present metals as beneficial to biodiversity. While this species indeed seems to thrive, the

overall invertebrate diversity has been shown to decrease under metal pollution since most species are far less able to adapt (Bengtsson & Rundgren 1984; Read *et al.* 1998; Lock et al. 2003; but see Creamer *et al.* 2008). Moreover we did not study effects on a community level, nor considered the food web in which Lycosids are involved (as in the framework proposed by Clements & Rohr, 2009). Spiders as macroconcetrators of metals most likely will impact their predators, through effects of Cd through the food chains and their impact on for example different species of invertebrates is inconsistent (Burger 2008).

Moreover, the effects of metals can neither be extrapolated to other stressors as their exposure, biological pathway and effects may significantly differ.

2 FUTURE PROSPECTS

2.1 Study populations and sample size

The results of research already performed and described in this study could be further confirmed, enlarged and refined studying more populations (replicas) and more individuals per population. Datasets for *P. saltans* now are not too small but larger dataset would provide more sound conclusions to be drawn. Initially more populations were sampled but after detailed species identification some populations were shown to be the closely related *Pardosa lugubris* or intermixed populations of both species. Because species differences obviously can interact with the results we decided only to study pure *P. saltans populations.* A better design future for *P. saltans* would have been to study at least two extra populations (heavily polluted and a reference) in the Kelmis geographical cluster and to include all eight populations in the life history study. This could also make the trends now observed for reproductive timing more convincing. A minimal sample size of 30 individuals would be preferable. For the genetic studies an even larger number of replica would allow for more sound results. For the study on *P. piraticus*, two populations were used that also differ in many other environmental factors, the one (Damvallei) being a quite stabile freshwater

marsh, and the other (Galgenschoor) a tidal brackish marsh. This implies that other factors than metal pollution could have affected mate choice. The use of more replica populationss would solve this issue.

Another improvement would be to complement the results with additional field studies, especially with regard to mate choice. Laboratory studies on sexual behavior are valuable because they are often the only possibility to study mating behavior and offer opportunities for precise measurement and experimental manipulation. Unfortunately, the laboratory environment is highly artificial compared to the natural situation where evolution actually occurs (Arnold *et al.* 1996). Moreover, potential mates are already brought together in a test arena which excludes mate search behavior. Yet, because of the hidden life style of *Pirata piraticus*, field mate choice experiments would be rather cumbersome. For *Pardosa saltans*, which is very active and occurring at high densities, it would be attainable, though demanding considerable effort. In anticipation of future field studies, we conducted a pilot study on mate choice in *Pardosa saltans* in the field, where marked males were exchanged between populations and the number of matings was counted. *P. saltans* is highly suited for this experimental work as couples remain in copula for several hours. Still, despite the high population densities and activity levels of the males, we were unable to recover a large number of resightings within the mating arena. More specifically, we only found 26 copulating males (108 mating pairs) to be marked out of a total of 878 marked males from four populations(D. Eraly, unpublished results). This did not allow us to make firm conclusions on the effect of population origin on mating probability.

2.2 Structural Equation Modeling

For the study on the different dependent variables related to mate choice (copulation probability, latency to court, latency to copulate, courtship duration, copulation duration) and the different independent variables, especially in the context of choice experiments (population of the female, population of the male, size of both partners, and the traits of the male not chosen) traditional statistical models become too complex and power too low to draw firm conclusions. Structural Equation Modeling would provide a solution to capture the complexity and allow conclusions to be

drawn. This is a multivariate statistical method that models causal relationships by placing them in a network. It allows to take into account measurement errors and consider both direct and indirect effects of variables in both directions. Central are latent variables that are theoretical concepts that unite different underlying mechanisms. They are not measured directly but are expressed in terms of one or more directly measurable variables called indicators. The goal is to propose and test a model that describes this underlying mechanism as good as possible. It is a combination of path analysis, factor analysis and regression analysis using maximum likelihood estimation techniques drawn (Bollen 1989; Johnson et al. 1991; Malaeb et al. 2000; Sih et al. 2002).

2.3 Other aspects of reproductive isolation

In the current study, we focused on size as a sexual selection trait influenced by natural selection, though we recognize other effects of stress treatment could possibly influence mate choice. Chemical cues will very likely differ (Clark *et al.* 1997; Fisher & Rosenthal 2006; Hoefler 2007) and male display behavior is also believed to be sensitive to nutritional state (Kotiaho 2000). Further research could provide more insight into the influence of food stress on these factors. Now we have observed that mate choice was affected by population origin, this renders a more detailed study of the traits involved much more worth the effort. It would also be interesting to study courthsip behavior and secondary sexual characteristics in *P. saltans* in more detail and compare this between polluted and reference populations. Another important factor are pheromones, which play an important role in mate searching, and how they are effected by stress (Gaskett 2007; Husak & Moore 2008; Rypstra *et al.* 2009; Jiao Xiaoguo *et al.* 2011).

Our research focused on premating reproductive isolation and more specific female mate choice. However, after copulation there still is a plethora of mechanisms possible to lead to reproductive isolation (postmating). A large amount of data is available from the laboratory breeding of both species. From the mating trials of *P. piraticus* from the four populations (chapter 4) of a part of the females cocoons were weighed and fecundity measured, for the others females were kept alive with their cocoons and

more than 2000 young were raised in the laboratory allowing to analyze fecundity, hatching time, survival, time until reaching adulthood. On their turn, they were mated and their own reproductive output was measured. Though a lot of young were raised, relatively high mortality rates in the F1-generation and a large number of possible mating types provided data that were too limited to allow clear conclusions.

2.4 Ecotoxicogenetics taken a step further

For the research question regarding genetic differentiation it would provide interesting to add a more explicit landscape context (North *et al.* 2011; Hanski 2012). Now we only found indirect evidence for the absence of geographic effects on genetic patterns. AMOVA showed no significant differentiation in the AFLP data between geographical clusters while comparing the clusters of polluted versus reference populations, differentiation does was present. In the outlier loci detected by Bayescan none seemed to be related to geographical cluster and the SAM analysis did not show significant correlation with location.

Moreover, comparing the results with the closely related *P. lugubris,* that also occurs on polluted sites and *P. piraticus* would gain insight whether similar mechanisms are deployed in related species. For *P. lugubris* the same AFLP protocol has already been executed on both polluted and reference populations, together with *P. saltans*. However, the data still need to be analyzed. It would be interesting to see whether the same outlier loci will be detected by Bayescan and SAM. If this is the case it would certainly be worth the effort to sequence these bands and to screen more populations and individuals for these alleles and to obtain insight into the evolutionary history and demography of these alleles. For the other study species *P. piraticus*, discussed in chapter 4, it would be interesting to conduct the same study.

However, with the AFLP technique we applied it is not possible to sequence the fragments that exhibit signatures of diversifying selection (Bensch & Akesson 2005). This hampers the identification of the genes involved in adaptation and, hence, to translate these results into a functional context.

Although the raise of powerful molecular tools enabled evolutionary biologists to hunt for genes involved in adaptation, the genetic mechanisms that lead a population towards a phenotypic optimum still remains largely elusive (Orr 2005). For instance, it is yet poorly understood whether divergence is caused by only a few genes of large effect or many genes with moderate to small effect (Orr 2005; Michel *et al.* 2010) whether adaptations and the evolution of new phenotypes are mainly the result of mutations in coding regions of the genome or rather of differences in gene expression (West-Eberhard 2005; Van Straalen & Roelofs 2006; Hoekstra & Coyne 2007; Steiner et al. 2007); if adaptation is caused by independent mutations or rather by introgression of standing genetic variation (Barrett & Schluter 2008) or the release of cryptic genetic variation by changes in epistatic interactions (Gibson & Dworkin 2004; Le Rouzic Arnaud *et al.* 2007). Such information is crucial to understand the mechanisms behind divergence under high levels of gene flow and the role of standing genetic variation in facilitating adaptive divergence. With regard to genes related to metals, though, recently some important insights were gained, mainly through the genomic approach. The mechanism of altered *cis*-regulation, trans-acting factors and multiple gene adaptation related to MTLP in *Orchesella cincta* (Janssens *et al.* 2009; Van Straalen Nico M. *et al.* 2011) were shown to play an important role in micro-evolution.

More insight into the genes that are effectively involved in adaptation can be attained by identifying the genes that are differentially expressed between contaminated and reference populations under both exposed and unexposed conditions. In particular in absence of genome information, this can be achieved by Suppression Subtractive Hybridization (SSH) (Diatchenko *et al.* 1996). SSH is a method that is used to generate a cDNA library that is enriched with cDNA's that are upregulated in a target sample (e.g. originating from a contaminated population) compared to a driver sample (e.g. cDNA library originating from a reference population). This method has been proven to be effective in identifying the genes involved in metal tolerance (Roelofs *et al.* 2007; Roelofs *et al.* 2009). It first normalizes the cDNA's of a target population and subsequently removes those cDNA's that are common in both target and driver population samples (Diatchenko *et al.* 1996). A pilot experiment on a subset of 80

spiders from the 6 populations reared in the laboratory from birth under differential exposure for the study described in chapter 2 were used for this analysis, followed by a subsequent preliminary verification by means of expression profiling of a candidate set of potentially differentially expressed genes. This work was conducted by Dr. Dick Roelofs and Janine Mariën at the Institute of Ecological Science, Free University, Amsterdam, The Netherlands.

Based on the individuals from the different populations of *P. saltans* that were bred in the laboratory under both Cd-exposed and control conditions (see chapter 2 for detailed information on the breeding design), three different SSH experiments were conducted i.e. (i) Cd exposed reference (target) population versus non-exposed reference (driver) population (ii) Cd exposed (target) contaminated population versus non-exposed (driver) contaminated population and (iii) unexposed contaminated (target) versus unexposed reference (driver) population. Enriched samples were cloned and 96 EST's were sequenced per sample. Although BLAST searches of these sequences did not reveal genes that unambiguously function as a metal binding protein such as metallothioinein, five genes showed a strong homology to genes that were also found to be involved in cadmium tolerance of the springtail *Orchesella cincta* (Roelofs *et al.* 2009) i.e. Innexin (XP_002433628), Troponin C (ABX75382), GTP binding protein (XP_002414923), 40S R ibosomal protein (ABX75476) and subunit 4 of NADH dehydrogenase (YP_025744). Higher expression of the latter two genes could indicate potentially higher energy demands as a result of cadmium detoxification. One of the upregulated genes showed strong homology with Scavenger receptor cysteine-rich protein (XP_001189615). The cysteine rich residues of this protein could potentially function as binding sites for Cd and Zn.

Given that the SSH procedure is sensitive for false positives and that the degree of differential expression is difficult to quantify, EST expression was further assessed by RT-PCR on the genes Troponin C and Scavenger receptor on 5 adult females of each treatment – population combination. This revealed significant upregulation of Troponin C in response to the Cd treatment ($F_{1,16}$=7.54; P = 0.014), which was not different among

populations (pop x treatment interaction $F_{1,16}$=1.17; P = 0.3). Upregulation for Scavenger receptor appeared to differ between both populations, with a significantly higher upregulation in the contaminated population compared to the reference population (pop x treatment interaction: $F_{1,16}$=4.73; P = 0.046).

Although it would be interesting to test if these results still hold when more populations are tested for the expression of these genes in response to contamination history, these results already indicate that exposure to cadmium could involve many genes, including these involved in general metabolic pathways, but also that long term metal exposure leads to an improved ability to induce adaptive plastic responses related to metal exposure. Its results also indicate that differentiation due to metal exposure is present in the study species.

Although SSH seems indeed promising to extract the genes involved in adaptation, current advancements in next-generation sequencing provide almost unrestricted availability of genetic information at both the transcriptome, and even genome level. Nowadays genome studies on non-model species are available at lower cost and with greater computing capacity, rendering the search for functional genes related to stressors a more straightforward task (Van Straalen & Feder 2012) (Roelofs *et al.* 2008). It allows (i) to identify genes that are responsible for ecotypic divergence, (ii) investigate footprints of selection for these genes, (iii) estimate their degree of linkage (Jung *et al.* 2007) (iv) investigate if recurrent adaptation is mainly due to standing genetic variation an (V) clarify evolutionary relationships among genes from different species.

One of the most important techniques in the light of this research would be to compare expression profiles between exposed and unexposed populations, as has been conducted for the springtail Orchesella cincta (Roelofs et al. 2009). This enables researchers to detect differentially expressed genes, and moreover to depict differences in frequency of single nucleotide polymorphisms in their coding region simultaneously (Stapley et al. 2010; Rice *et al.* 2011; Wheat & Vogel 2011; Van Straalen & Feder 2012).

At the population genetic level, the comparison of differentiation levels at a large number of genes, as conducted by means of AFLP markers in the current study, could be replaced by a genotype sequencing or RAD sequencing approach (Davey *et al.* 2011). A major advantage of this technique is that sequence information of the markers that exhibit signs of selection are immediately available. In combination with transcriptome or, even better, genome sequence information, candidate genes can be identified and a more functional interpretation can be given.

The new approach to genomics no longer searches for single gene mutations but takes a more holistic approach on the genome and analyzes as many genes as possible, their transcripts, their regulation, the way they interact and the proteins they encode. This techniques even allows metagenomics, the search for common genetical mechanisms in different organisms inhabiting the same environment (Van Straalen & Feder 2012).

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Never let go of
what you strive for
Hold tight,
never let go
Never think
you can't make
a change

Hatebreed

SUMMARY

The strength and direction of natural selection may differ in space and time, and may lead to adaptation and differentiation of populations both at the phenotypic and genotypic level. The exposure of organisms to different pollutants is widespread but a large number of species still inhabit heavily polluted environments. Metals are one of the most persistent pollutants with strong impacts on fitness and generally considered to induce a strong selection pressure. Yet, organisms have several mechanisms by which they can protect themselves against the adverse effects of metals. Particular enzymes and molecules can increase tolerance, with a major role for MetalloThionein-Like Proteins. Moreover, populations can also have the ability to physiologically acclimate, allowing to deal with environmental unpredictability.

One of the possible consequences of local adaptation with potentially great evolutionary impact is the evolution of reproductive isolation between adapted and non-adapted populations, both through direct and indirect processes. Mate choice may diverge if adaptation causes phenotypic traits on which mate choice is based to diverge due to natural selection ("by-product mechanism") like size-assortative mating and a shift in reproductive timing. Condition-dependence in female mate choice is an important factor since this costly behavior is also expected to depend on her reproductive status.

Moreover, we also expect effects on population genetic parameters through metal exposure, with possible effects on genetic variation, an increase in genetic differentiation between exposed and unexposed populations and the occurrence of specific loci linked to metal pollution, detected as "outlier loci".

The topics discussed above are studied on the wolf spiders (Lycosidae) *Pardosa saltans*, inhabiting forest margins, and *Pirata piraticus*, a species from wet open habitats. Spiders are strong accumulators of metals and their internal concentration therefore reflects the exposure throughout their lifetime.

As expected for life histories of organisms facing energetic constraints, adult size and condition correlated negatively and egg mass positively with Cd concentrations in field populations of *P. saltans*. In the population that showed the highest cadmium and zinc body burdens, reproductive output and allocation were lowest and reproduction was postponed. Contrary to our expectation, MTLP concentrations did not increase in exposed populations, indicating that this defense mechanism cannot explain the observed variation in life histories. Lack of an increase in MTLP concentration under increased metal stress contrasts with results obtained from an experimental exposure study that showed elevated MTLP concentrations, however in absence of measurable effects on individual growth and survival.

Based on AFLP markers we found larger genetic differentiation between polluted and reference populations in *Pardosa saltans* than between populations within these groups, but no evidence for genetic erosion, i.e. an overall decrease of genetic variation, due to metal exposure. Several outlier loci were detected that are linked to metal exposure and need further investigation to define the genes involved. The results from these analyses not only revealed signs of genetic differentiation indicative of local adaptation, but moreover suggest that metal pollution in this metapopulation selects identical alleles in the face of homogenizing gene flow.

Effects of metal adaptation and food stress on mate choice was studied in *Pirata piraticus* spiders. Compared to control females, food-stressed females from the reference population showed a decreased probability of copulation and preferred smaller mates. Females from the polluted population, in contrast, did not show a significant response to food stress and showed size-assortative mating, most strongly under food stress. Results from this study support the prediction that variation in body condition, driven by local ecological factors, may affect mating behavior and may ultimately lead to population divergence in important life history traits such as body size.

Evidence for local adaptation to metal exposure by altered life history traits or physiological adaptations seems rather weak in *Pardosa saltans* However, genetic differentiation and presence of outlier loci support a pattern of genetic divergence linked to metal exposure. Integration of the results did not appear to be straightforward, which is most probable due to the expression of adverse effects if they exceed a particular threshold and synergistic effects of metal pollution may be more important than trade-offs with physiological defense mechanisms in shaping life history traits in field populations. Hence, this study demonstrates that investigating the effect of metal stress should involve multiple approaches.

SAMENVATTING

De intensiteit en richting van natuurlijke selectie kan variëren in de ruimte en de tijd en kan leiden tot natuurlijke adaptatie en differentiatie van populaties, zowel fenotypisch als genetisch. De blootstelling van organismen aan verschillende verontreinigende stoffen is wijdverbreid, maar een groot aantal soorten kan toch overleven in deze sterk vervuilde omgevingen. Metalen zijn één van de meest persistente polluenten die een sterke invloed hebben op de fitness en er wordt in het algemeen aangenomen dat ze een sterke selectiedruk betekenen. Verschillende soorten hebben echter mechanismen ontwikkeld om zich tegen de negatieve effecten van blootstelling aan metalen te beschermen. Bepaalde enzymen en moleculen kunnen de tolerantie verhogen, waarbij Metallothioneïne-achtige Proteïnen (MetalloThionein-Like Proteins) een belangrijke rol spelen. Bovendien zijn bepaalde populaties ook in staat om zich fysiologisch aan te passen (acclimatie) om op deze manier om te gaan met variatie in het milieu.

Eén van de mogelijke gevolgen van lokale adaptatie met een potentieel grote evolutionaire impact is de evolutie van reproductieve isolatie tussen geadapteerde en niet-geadapteerde populaties, zowel door directe als indirecte processen. Partnerkeuze kan divergeren als door adaptatie de kenmerken waarop de partnerkeuze gebaseerd is divergeren door natuurlijke selectie (het zogenaamde “bijproduct mechanisme”), zoals het assortatief paren volgens lichaamsgrootte (“size-assortative mating”) en een verschuiving in het tijdstip van de voortplanting. Conditie-afhankelijkheid van de keuze van een partner door vrouwelijke individuen is hierbij een belangrijke factor aangezien deze ook afhankelijk is van haar reproductieve status.

Bovendien zullen ook de populatiegenetische parameters beïnvloed worden door vervuiling met metalen, met mogelijke effecten op genetische variatie, toegenomen genetische differentiatie tussen wel- en niet vervuilde populaties en het voorkomen van aan metaalvervuiling gelinkte loci gedetecteerd als “outlier” loci.

De onderwerpen die hierboven behandeld worden, werden bestudeerd aan de hand van twee soorten wolfspinnen (Lycosidae), *Pardosa saltans*, die in bosranden voorkomt en *Pirata piraticus*, een soort van vochtige open habitats. Spinnen zijn sterke accumulatoren van metalen en hun interne concentratie weerspiegelt dan ook de blootstelling gedurende hun levensduur.

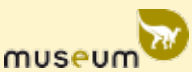
Zoals verwacht voor organismen die blootgesteld worden aan metalen, met de bijhorende energetische beperkingen, correleerde volwassen lichaamsgrootte en conditie negatief en de grootte van de eitjes positief met Cd concentratie in natuurlijke populaties van *P. saltans*. In de populatie met de hoogste concentratie aan cadmium en zink was de reproductieve output ook het kleinst en was er een trend naar het uitstel van de voortplanting. In tegenstelling tot de verwachtingen waren de MTLP concentraties niet hoger in de vervuilde populaties. Dit wijst er op dat dit verdedigingsmechanisme de geobserveerde variatie in levensgeschiedenis-kenmerken niet kan verklaren. Het feit dat er geen toename in MTLP concentratie bij grotere metaalblootstelling kon vastgesteld worden staat in contrast met de resultaten van de laboratoriumstudie waarbij de spinnen die blootgesteld werden aan Cd een hogere MTLP concentratie hadden. Dit had echter geen negatieve impact op hun individuele groei of overleving.

Op basis van AFLP merkers vonden we een grotere genetische differentiatie tussen metaalvervuilde- en referentie-populaties van *Pardosa saltans* dan binnen deze groepen, maar geen bewijs voor genetische erosie (een algemene afname van de genetische variatie) door blootstelling aan metalen. Verschillende outlier loci gelinkt aan metalen werden waargenomen en verder onderzoek is nodig om de betrokken genen te identificeren. De resultaten van deze analyses toonden dus niet alleen tekenen van genetische differentiatie, die wijst op lokale adaptatie, maar suggereren ook dat metaalvervuiling in deze metapopulaties identieke allelen selecteert in een systeem van homogeniserende genenuitwisseling.

Effecten van adaptatie aan metalen en voedingstress op partnerkeuze werden bestudeerd bij *Pirata piraticus.* In vergelijking met de vrouwtjes van de controlegroep, vertoonden de gestresseerde vrouwtjes van de referentiepopulatie een lagere kans om te paren en verkozen ze kleinere

mannetjes. Vrouwtjes van de vervuilde populatie daarentegen vertoonden geen significante respons op de voedingstress en vertoonden een patroon van assortatief paren volgens lichaamsgrootte. Dit patroon was nog sterker onder stress. De resultaten van deze studie bevestigen de voorspelling dat variatie in conditie, bepaald door lokale ecologische factoren, het voortplantingsgedrag kan beïnvloeden en uiteindelijk kan leiden tot divergentie tussen populaties in belangrijke kenmerken als lichaamsgrootte.

Bewijs voor lokale adaptatie aan metaalblootstelling door veranderingen in levensgeschiedeniskenmerken of fysiologische adaptatie is eerder zwak bij *Pardosa saltans.* De patronen van genetische differentiatie en de aanwezigheid van outlier loci daarentegen bevestigen een patroon genetische divergentie ten gevolge van metaalblootstelling. De integratie van de verschillende resultaten bleek niet voor de hand liggend, wat waarschijnlijk te wijten is aan de aanwezigheid van bepaalde drempelwaarden voor negatieve effecten zichtbaar worden en de synergetische effecten van metaalvervuiling zouden belangrijker kunnen zijn dan trade-offs met fysiologische verdedigingsmechanismen in het bepalen van de levensgeschiedeniskenmerken in vervuilde populaties. Deze studie toont dus het belang aan van meervoudige benaderingen in het bestuderen van de impact van metaalstress op natuurlijke populaties.



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